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STUDIES ON COLD-SMOKED FISH PRODUCTION AND THEIR RELATION TO CONTROLLING SPOILAGE AND SAFETY

Thesis submitted to Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology, with specialization in Microbiology

Maria Manuela Ramos Vieira da Silva

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“Success is not final, failure is not fatal,
it is the courage to continue that counts”
(Wirston Churchill)

Abstract

Smoked fish products are currently part of the Portuguese diet, being accessible to a large group of people. They are presented on Portuguese commercial point of sale packed in a vacuum, in small sliced pieces or in the form of a fillet. Fishes such as salmon, salmon-trout and swordfish, with European provenance, are used as raw material for the cold smoking production. The shelf-life depends on the type of cold smoking applied, ranging from 2 to 6 weeks at refrigeration temperatures ≤5ºC. Microbiological and physicochemical characteristics have demonstrated the dominance of Lactic Acid Bacteria, *Enterobacteriaceae*, and vibrios, among others, with the possibility of other groups of microorganisms being present, such as the pathogen *Listeria monocytogenes*. Attempts to create a quality index of cold-smoked fish products have been the subject of several studies in order to establish a correlation between the useful shelf-life of the product and the physicochemical, sensorial and microbiological characteristics.

The main objective of this research was to characterize the microbial ecology of vacuum packed cold-smoked fish available in a Portuguese commercial point of sale and at a pilot-scale production by the identification of determinant variables, which influence the microbiological and physicochemical quality of the cold smoked fish. The investigation was conducted to characterize commercial cold-smoked fish products available in the Portuguese market, mainly salmon (*Salmo salar*) and salmon trout (*Oncorhynchus mykiss*), considering microbiological and physicochemical studies. From microbiological characterization, the ability of different bacteria isolated from cold-smoked fish to produce biogenic amines was evaluated, using different descarboxylation agar growth medium. A pilot-scale cold smoking controlled experiments using salmon trout were conducted to study: a) the effect of the application of ozone as a disinfected agent on whole fresh fish and fillets on reduction of microorganisms, including *L. innocua*; b) the effect of a previous freezing step (-20°C) of individual samples of vacuum packed cold-smoked salmon trout before product commercialized at chilled storage on microbial ecology c) the effect of combined treatments of salting/drying/smoking (wet or dry salting, addition of sugar in the salting mixture and long and short smoking) on microbiological and physicochemical properties followed by the use of vacuum and modified atmospheres packaging chilled storage conditions at 5ºC.

Results from samples obtained at the Portuguese point of sale evidenced differences on shelf-life products, as well as on microbiological numbers of the cold-smoked samples. Some of the cold-smoked samples would be at the limit of the allowed microbial load for RTE products, even before reaching shelf-life´s limit. The results also suggested the decrease in coefficient of variation of samples for aerobic plate counts and for numbers of *Enterobateriaceae* in controlled time and temperature laboratory conditions. The results demonstrated the suitability of growth culture medium on selection of bacteria producing biogenic amines. Some bacterial strains belonging to the group of LAB and *Enterobacteriaceae* were positive for tyramine production and less for histamine production. Complementary results obtained from HPLC determinations, showed higher concentration of tyramine production by *Carnobacterium divergens* and by *Lactoccocus lactis lactis*.

From gaseous ozone treatments applied to whole and fresh fillets salmon trout, a decrease of less than $1Log_{10}$ in *L. innocua* (as a surrogate for the pathogen *L. monocytogenes*) numbers occurred on ozone treated samples in all sampling occasions. Aerobic Plate Count was slightly lower on fresh fillets after treatment and during three weeks of storage. From ozone treatments applied to whole fresh fish, a reduction greater than 1 Log_{10}/g of *L. innocua* occurred on smoked samples at the end of the storage period. These results were more pronounced when the slime present on the whole fish surface was removed. From a previous freezing step (-20ºC) the results showed an general effect on microbial load throughout storage of previously frozen samples at initial stage of chill storage (1^{st} week) for total aerobic plate count, LAB and H_2S producing bacteria, as compared to non-frozen cold-smoked samples only chilled. A significant increase in numbers of H_2S-producing bacteria was observed in previously frozen samples, independently of the type of salting applied. *Enterobacteriaceae* group was less affected by the previously freezing step. DSC thermograms showed changes in muscle structure after salting/cold-smoking process. The stability of myofibrillar proteins were affected by salting/smoking treatment and the additional freezing step can result in decrease in product quality. Results from combined treatments of salting/drying/smoking on production of cold-smoked salmon trout revealed that wet salting treatment (especially for shorter salting times) did not produce the same results on microbiological and physicochemical characteristics on the end product, compared with dry salting. Overall, dry salting is preferable to brining for reducing microbial growth in cold-smoked salmon trout stored in

VP. Higher sugar content in the salting mixture (salt:sugar|3:1) induced an increase in microbiological numbers. The smoking process characterized by long drying and short smoking times (Group II – Dry 6h and Smoke 2h) encouraged a general increase in microbiological numbers of cold-smoked samples, with a significant increase in LAB counts, but a negative effect on the samples regarding microbiological quality, with significant increase in *Enterobacteriaceae* and H_2S-producing bacteria. After 3 week storage the average of samples presented levels of trimethylamine (TMA) (up to 30 mg in 100 g of fish). A positive effect of short dry and long smoke exposure on microbial ecology was observed (Group I – Dry 2h and Smoke 6h) in cold-smoked fish, dry salted and packaged either in VP or MAP. MAP represents an alternative of packaging to VP, reducing the microbiological activity of some spoilage bacteria.

The present research highlights how essential is the improvement of careful control in three areas of work that compose the production of cold smoked fish: (1) the control of quality of the raw material; (2) the design of technological procedures applied during the curing/smoking preservation process and (3) type of packaging and chill storage conditions. Advances in the integrated production of cold smoked fish using the preservative combined effects as the 'hurdle concept' should be applied defining technical procedures to product stability and safety commercialization.

Resumo

Os produtos da pesca fumados fazem atualmente parte da alimentação dos portugueses, estando acessível a um grande grupo de pessoas. Apresentam-se nas superfícies comerciais portuguesas embaladas a vácuo, em peças pequenas laminados, e ou em forma de filete. Peixes como o salmão, a truta salmonada e o espadarte, com proveniência europeia, são utilizados como matéria-prima para a fumagem a frio do pescado. A vida de prateleira depende do tipo de fumagem a frio aplicado, variando entre 2 a 6 semanas, armazenado a temperaturas de refrigeração <5ºC. As características microbiológicas e físico-químicas têm demonstrado a dominância de Bactérias Ácido-Lácticas, *Enterobacteriaceae*, e vibrios, havendo a possibilidade de outros microrganismos estarem presentes como é o caso de *Listeria monocytogenes*, afetando a qualidade e a segurança destes produtos. Nos últimos anos, a tentativa de criar um índice de qualidade de peixe fumado a frio foi alvo de vários estudos, com o objetivo de estabelecer uma correlação entre a vida útil do produto e as características físico-químicas, sensoriais e microbiológicas.

A presente investigação teve como principal objetivo a caracterização da ecologia microbiana de peixe fumado a frio disponível no mercado português e à escala piloto, através da produção de peixe fumado a frio, identificando determinadas variáveis com influência na qualidade microbiológica e química destes produtos. Numa primeira fase da investigação foi efetuada a caracterização microbiológica dos produtos de pescado fumado a frio disponíveis no mercado português, essencialmente salmão (*Salmo salar*) e truta salmonada (*Oncorhynchus mykiss*). Relativamente à caracterização microbiológica, bactérias isoladas de salmão e truta salmonada foram testadas para a produção de aminas biogénicas, tiramina e de histamina, utilizando meios de cultura específicos de crescimento. Estudos à escala piloto sobre o processo de fumagem a frio de truta salmonada foram conduzidos, com objetivo de estudar: a) o efeito da aplicação do ozono, enquanto agente desinfetante em filete e peixe inteiro de truta salmonada fresca na redução de microrganismos viáveis totais e *L. innocua*; b) a aplicação de um passo prévio de congelação (-20ºC) em amostras individuais de truta salmonada embalada a vácuo na ecologia microbiana do produto; c) o efeito de tratamentos combinados de salga/secagem/fumagem (salga seca e húmida, adição de açúcar na mistura da salga,

duração curta e longa de fumagem) e embalamento a vácuo ou em atmosferas modificadas nas características físico-químicas e microbiológicas do produto final.

Resultados sobre a classificação das amostras comerciais de peixe fumado a frio embalado a vácuo, demonstraram variabilidade das amostras, pelas diferenças nos períodos de vidas de prateleira e características microbiológicas. Algumas amostras apresentavam já estar muito próximo dos limites de rejeição estabelecidos para produtos prontos a comer, antes de terminar a vida de prateleira. Os resultados evidenciaram que em condições controladas de tempo e temperatura, houve uma diminuição do coeficiente de variação nas amostras, e para o número de microrganismos aeróbicos totais e grupo *Enterobacteriaceae*. Sobre o resultado da pesquisa de estirpes produtores de aminas biogénicas em condições específicas em meio de cultura, os resultados indicaram a habilidade de algumas bactérias LAB e *Enterobacteriaceae* produzirem tiramina e menos a histamina. Resultados complementares utilizando HPLC para a quantificação das aminas, mostraram níveis elevados de tiramina produzidos pelas bactérias *Carnobacterium divergens* e *Lactoccocus lactis lactis*.

Os resultados envolvendo tratamento com ozono gasoso em filetes e em peixe inteiro fresco, mostrou um decréscimo inferior a $1Log_{10}$/g de *L. innocua* em amostras tratadas com ozono em todas as experiências. Contagens totais de microrganismos viáveis foram baixas no peixe fresco e durante a armazenagem a frio ao final de três semanas. Uma redução superior a $1Log_{10}$/g de *L. innocua* foi observada em peixe tratado no final do período da armazenagem. Quando retirado o *slime* da superfície do peixe, o efeito de redução foi mais pronunciado.

Relativamente ao tratamento prévio da congelação (-20ºC) aplicado em amostras individuais fumadas a frio de truta salmonada embalada a vácuo, os resultados evidenciaram um aumento da carga microbiana nas amostras previamente congeladas na primeira semana de armazenamento em refrigeração, essencialmente para as bactérias aeróbias totais, LAB e bactérias produtoras de H_2S. Um aumento significativo para bactérias produtoras de H_2S foi observado, independentemente do tipo de salga a que foram sujeitos. O processo prévio do passo da congelação pareceu ter menos efeito no grupo *Enterobacteriaceae*. Alterações na estrutura das proteínas musculares da truta-salmonada após o processo de salga/fumagem, foram evidenciadas nos termogramas obtidos por *Differencial Scanning Calorimetry* (DSC). A estabilidade das proteínas

miofibrilares foram afetadas pelo processo da salga/fumagem e o processo adicional de congelação poderá afetar a qualidade microbiológica da truta-salmonada fumada a frio.

Os resultados obtidos sobre os tratamentos combinados da salga/secagem/fumagem revelaram que a salga húmida e salga seca apresentaram diferentes efeitos nas características físico-químicas e microbiológicas do produto final. Na generalidade, a salga seca apresentou efeito maior na perda de peso (menor rendimento do processo), e melhor desempenho na obtenção de teores de sal em fase aquosa, havendo efeito no controlo/redução do crescimento microbiano. A presença de maior teor de açúcar na mistura com sal (sal:açucar|3:1) induziu um incremento no crescimento microbiano nas amostras em geral, quando comparado com a mistura (sal:açucar|5:1).

Relativamente ao efeito da fumagem, os resultados indicaram que a combinação 6h de secagem e 2h de fumagem (Grupo II) induziu um crescimento significativo de bactérias ácido lácticas no produto final, com efeitos similares em outros microrganismos, como o grupo *Enterobacteriaceae* e bactérias produtoras de H_2S. Ao final de três semanas de armazenamento em refrigeração, foram registadas um aumento do teor médio de trimetilamina (TMA) (superior a 30 mg. em 100 g de peixe). Comparativamente, o tratamento combinado de 2h de secagem e 6h de fumagem (Grupo I) mostrou ser mais eficaz no controlo do crescimento microbiano, em amostras tratadas por salda seca (8h) e embaladas a vácuo ou em atmosferas modificadas. O embalamento em atmosferas modificadas representa uma alternativa reduzindo a actividade microbiana de alguns microorganismos degradativos.

Genericamente o presente estudo evidencia a necessidade do controlo e implementação de procedimentos controlados no processo de produção de fumagem a frio de pescado, considerando (1) A qualidade microbiológica e química da matéria-prima; (2) A definição do processo tecnológico a aplicar (descrição, objetivos e características produto final) e (3) Tipo de embalamento e controlo das condições de armazenagem. A criação de processos de fumagem a frio de peixe baseados em '*'hurdle concept technology*' poderão através da sinergia dos agentes de preservação constituir uma solução à estabilidade e comercialização em segurança destes produtos.

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KEYWORDS

Cold-smoked fish

Shelf life

Cold-smoking process

Microbial ecology

Salmon

Salmon-trout

Spoilage bacteria

Vacuum package

Modified Atmospheres Package

Biogenic Amines

Trimethylamine

Quality

Safety

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LIST OF SYMBOLS AND ABBREVIATIONS

APC- Aerobic Plate Counts

Aw – Water Activity

CFU- Colony Forming Units

CSS – Cold-smoked Salmon

EC – European Community

EU – European Union

EUMOFA - European Market Observatory for Fisheries and Aquaculture Products

FAO – Food Agriculture Association

FDA – Food and Drug Administration

HACCP – Hazard Analysis and Critical Control of Points

LAB – Lactic Acid Bacteria

LH medium – Long and Hammer medium

LPFP – Light Preserved Food Products

MA – Modified Atmospheres

MAP – Modified Atmospheres Package

NAP medium – Nitrate Actidione Polymyxin

pH – measure of hydrogen ion concentration (measure de acidity or alkalinity of a solution)

QI – Quality Index

QIM - Quality Index Method

TBAS – Thiobarbituric Acid Reactive Substances

TMA – Trimethylamine

VRBGA – Violet Red Bile Glucose Agar

WPS – Water Phase Salt

VP – Vacuum Packing

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CHAPTER 1

Introduction, Scope and Outline

1.1 Introduction

Salmon products form a traditional part of many European diets. The majority of salmon consumed in European Union (EU) is farmed, and salmon is the most consumed farmed species in the EU as well as the third most consumed fish species overall (European Market Observatory for Fisheries and Aquaculture Products - EUMOFA, 2016). Per capita consumption in the EU was over 2 kilograms (kg) per person in 2014 (EUMOFA, 2016).

The EU achieved approximately 18% self-sufficiency in salmon products in 2014, meaning that 82% of demand had to be imported (Sector Trends Analysis, 2017). Trade in salmon and other salmonids was the largest component of inter-EU trade, at 28% in 2015. In total, salmonid trade accounted for 6.4 billion euros. Salmon species alone accounted for 5.7 billion euros (EUMOFA, 2016). Salmon in the EU tends to be processed and consumed smoked, with France and Poland both producing large amounts of smoked salmon (EUMOFA, 2016). Nonetheless, fresh salmon is used in a variety of products. Spain is the leading market for fresh fish and seafood in the EU. However, its market has declined by a compound annual rate of 2.57% since 2012, while the UK's market grew over the same period. Considering the market share, Portugal is in fifth position, with 471.9 tonnes in year 2016, and a percentage of 8.37 (Sector Trends Analysis, 2017). Data from FAO (2014) reported that 12% of the fish was commercialized smoked, dried, cured or salted, and the other was used fresh and refrigerated (46%), frozen (29%) and transformed or canned (13%).

Production of smoked fish and especially smoked salmon is one of the most important sectors in European fisheries. Nowadays, it is a product of general consumption, as compared with a few decades ago when it was considered as a “luxury” food item. It is a

ready–to–eat (RTE) product whose demand had increased considerably in many European countries. Portugal accompanied the consumption of these products, however, in relation to internal production, has no current representation, having recorded the representative production between years 1999-2009, in most cases for export or for consumption in tourism areas of Portugal. Salmon (*Salmo salar*) and salmon-trout (*Onchorynchus mykiss*) were the elected species for this type of industry and this last are produced intensively in our country. Although the smoked fish production does not occupy a significant position in the Portuguese and one way to monetize this production will be increasing the added value that comes from the processing. According to the general directorate of fisheries, in relation to manufacturing industry, in year 2015, Portugal had two companies licensed in smoking fish. In comparison, the smoked meat products are widely produced in Portugal, as part of the traditional diet.

Cold-smoked fish products are available in the Portuguese commercial market imported from different European countries. Cold-smoked salmon (CSS) is typically sliced and vacuum packed before distribution as a chilled product. Processing includes salting followed by drying and smoking at 18–30°C. This result in a smoked product with 3–8% water phase salt (WPS) corresponding to water activity (aw) of 0.950-0.983 and a pH of 5.9-6.3 (Giménez and Dalgaard, 2004). However, such high NaCl concentrations should not be utilized due to consumer's health, and a combined effect of several components need be introduced to control and assure safety of cold-smoked products. Production and retailing of cold smoked fish are a complex multistep process that includes packaging in vacuum packaging (VP) or modified atmosphere packaging (MAP) and storage retail under refrigerated conditions (below 4°C) (FDA, 2001a). The shelf-life of the final product depends on numerous and interrelated production parameters such as characteristics of the raw material, process hygiene, salting method, salt level, smoking conditions, vacuum or

under modified atmosphere (MA) (Lovdal, 2015) and storing conditions (Silva and Gibbs, 2015).

Therefore, it is of great importance to optimize the individual processing steps involved in the production (Gallart-Jornet *et al*., 2007b). Decision taken by a producer regarding choice of smoked fish technology and related parameters to be used is influenced by the market demand and also by the need for economical profit. Furthermore, the choice of the process control parameters, such as duration of salting, concentration of brine or application of dry salt, and smoking time and temperature, also influences the specific characteristics of the final product that are desired (Cardinal *et al*., 2004).

In addition to salting and smoking effects, the shelf-life of smoked fish is affected by storage conditions. Differences in packaging and temperature related parameters throughout the storage period result in different product shelf-lives. Also, appropriate selection of raw material and salting of fish is the first step in the smoked fish production process, which is critical for the shelf life, good quality and yield of the final product (Acharyga, 2011; Birkeland *et al*., 2004b; Bjornevik *et al*., 2018). The preservative effect of salting is due to lowering of a_w (Birkeland and Bjerkeng, 2011; Iacumin *et al*., 2017; Jittinandana *et al*., 2002) and thus reducing the growth of many spoilage and pathogen microorganisms (Rørvik, 2000). Dry salting is traditional but still the most common method of fish salting, whilst wet salting is rare in practice. Nevertheless, it should be noted that, in modern times, fish products are relatively lightly salted. Both the salt content and the level of smoking applied vary among and within European countries (Røra *et al*., 2004; Gallart-Jornet *et al*., 2007a, 2007b) as a shelf-life remained (Silva and Gibbs, 2015). Storage temperature, a_w and WPS markedly influence the shelf life of VP-CSS and a

synergistic effect of NaCl and smoke components on shelf life has been reported (Dalgaard, 2002; Leroi and Joffraud 2000a, 2000b; Hansen *et al*., 1995).

From health perspective and due to the negative health implications of PAH, the importance of using liquid smoke condensates in food products as a replacement to traditional smoking was being studied (Ayvaz and Atar, 2016; Martinez *et al.*, 2012).

Spoilage is primarily due to microbial activity and at ≤10°C the spoilage microorganisms are dominated by lactic acid bacteria (LAB) sometimes together with *Photobacterium (Ph.) phosphoreum* or *Enterobacteriaceae* (Giménez and Dalgaard, 2004). Processing environment is a source of contamination of salmon fillets with *Pseudomonas* spp. and *Shewanella* spp. while *Photobacterium* spp. most likely originates from the live fish and seawater (Leroi and Joffraud, 2011; Moreto *et al*., 2016). Bacteria on the product can originate from the raw materials or be introduced during processing by e.g. cross contamination from equipment or by food handlers. In the past years, studies on recognition of spoilage microorganisms have been made with the proposal to achieve a quality index method for cold-smoked fish (Dalgaard, 2002; Jorgensen *et al*., 2000b; Leroi *et al*., 2001). LAB can reach their maximum population density rapidly and remain at this level during up to 50% of the products' shelf-life (Leroi *et al.*, 1998; Tomé *et al.,* 2006).

Processing of VP-CSS include recognizing the critical control point for *Listeria monocytogenes* and although cold-smoking seems to reduce numbers of the pathogen it is unlikely the product can be produced completely free of this pathogen (Gram, 2001a). *L. monocytogenes* can grow in naturally contaminated VP-CSS and this is of major importance for human health (EFSA, 2009, 2013). Smoke components and LAB, are important factors controlling growth of *L. monocytogenes* in VP-CSS (Lodval, 2015; Tocmo *et al.,* 2014; Tomé *et al*., 2008). Previous studies indicated that at ≤10°C, LAB

inhibited growth of *L. monocytogenes* in VP-CSS but it remains uncertain if spoilage microorganisms limit growth of *L. monocytogenes* at higher or in variable temperature storage conditions (Katla *et al.,* 2001; Ross *et al.,* 2000). In fact, the prevalence of *L. monocytogenes* in cold-smoked fish products at a retail level, is highly variable, representing in previous studies, a percentage of 15% to 40% (Gram, 2001a). After the year 2000, data reported a prevalence of 0% to 61%, with an average of 9.8% (Lovdal, 2015). EFSA (2013) published an EU baseline survey on the prevalence of *L. monocytogenes* in certain ready-to-eat (RTE) foods, from to 2010 until 2012 (Portugal did not participate). The results of the survey showed a prevalence of 10.4% for fish samples (cold-smoked, hot-smoked or graved fish) at the time of sampling and 10.3% at the end of shelf-life (20-60 days). At the end of shelf-life, 1.7% of contaminated fish samples with *L. monocytogenes* exceeded 100 cfu/g. and 1% at the time of sampling.

1.2 Scope and Outline

Cold-smoked fish are available in the all retail markets in Portugal and other EU countries, representing a traditional production in many EU countries. Risk associated to unsafe products to consumers and their relation to the presence of *L. monocytogenes* was a concern of many authorities. Thus, the cold-smoked fish production processes are of great importance to achieve a very good product in quality and safety, concerning the microbiology and chemical composition. Several parameters associated to the cold-smoked fish production need be studied, such as, the previous procedures of salting/drying, before smoking, and after, packaging and storing conditions. The main objective of this research was to characterize the microbial ecology of vacuum packed cold-smoked fish available in a Portuguese commercial point of sale and at a pilot-scale production, by

identification of determinant variables related to smoking processing, packaging and storing conditions, which influence the microbiological and physicochemical quality of the cold smoked salmon-trout (*Onchorynchus mykiss*).

To achieve this main goal, specific objectives were defined:

1. Understanding the limit of shelf-life of cold-smoked fish available to consumer in relation to smoking process and chilled storage conditions.
2. Characterizing the microbial ecology and chemical aspects of chilled vacuum-packed cold-smoked fish with the focus on spoilage microorganisms and bacteria biogenic amine producers.
3. Introducing challenge studies on cold-smoked fish production and evaluate their impact on microbiological and chemical characteristics during chill storage:

   a) Investigate the effect of previous ozone treatment of raw fish fillets of salmon-trout before salting/drying/smoking, on the end-product;

   b) Investigate the effect of smoking processing on stability of myofibrillar proteins of salmon trout;

   c) Investigate the effect of a previous freezing step at -20ºC before distribution and retail of cold-smoked salmon-trout at chill conditions (5ºC) on microbial ecology of the end-product and,

   d) Investigate the effect of different combined treatments of salting/drying/smoking on microbial ecology of cold-smoked salmon-trout stored in VP or MAP;
4. Improving quality on cold-smoked fish production: good manufacturing practices and Hazard Analysis and Critical Control of Points (HACCP) implementation at production and at retail levels.

This thesis is structured in four parts which comprises 6 chapters. The different chapters are presented by the order, in general which the practical work was developed. **Part I** comprise **Chapters 1 and 2**, and include a general introduction, structure of the manuscript and literature review. Each step of cold-smoked fish process is discussed in detail. The influence of different steps of smoking process and selected parameters on the properties of the end-product is described. The microbiological contamination is introduced associated to different conditions and, characteristics of microbial ecology, spoilage bacteria and pathogens are described. The product shelf-life and microbiological quality are described.

Part II comprises **Chapter 3**, which include the studies on microbiological characteristics of cold-smoked fish samples and identification of bacteria biogenic amine producers (tyramine and histamine) isolated from commercial products. Microbiological quality of cold-smoked fish samples available on Portuguese market and from Portuguese smoked fish processing plants are characterized. Studies on microbial evolution during chill controlled storage, the coefficient of variability of samples and the remaining shelf-life are presented.

Part III encompasses **Chapter 4**, which describes the results of the challenge studies of cold-smoked fish production in a pilot-scale production. The smoking process was investigated and different macro processes were tested (salting methods, smoking process and type of packaging) in respect to their effects on physicochemical and microbiological characteristics of cold-smoked fish. Changes on muscle structure during smoking processing by the myofribrillar proteins patterns are presented. A previous treatment with gaseous ozone on raw fish fillets before salting/smoking is introduced in the process and a freezing step period on cold-smoked vacuum-packed salmon trout before

chill storage conditions are presented. Selected conditions related to combined treatments applied on salting/drying/smoking process are identified with greater potential to control spoilage.

Part IV comprises **Chapter 5** in which the main conclusions of this study are presented, and **Chapter 6** the outline proposals for future work.

The work presented in this thesis was reported in six papers: three papers published in peer-reviewed scientific journals, two already submitted and one, to be submitted in peer-reviewed scientific journal:

Chapter 2

Silva, M. V. and Gibbs, P. A. 2019**.** Cold-smoked salmon-trout quality: production, spoilage and microbiological contamination aspects (*in preparation to be submitted to Food Science and Technology International*).

Chapter 3

Silva, M. V., Pinho, O., Ferreira I., Plestilová, L., and Gibbs, P. A. 2002. Production of histamine and tyramine by bacteria isolated from Portuguese vacuum-packed cold-smoked fish. *Food Control* 13: 457–461.

Silva, M. V. and Gibbs, P. A., 2015. Significance of Biogenic Amines in Cold-Smoked Fish and Their Relation to Microbiological Characteristics of Products, Available in Portuguese Retail Markets. *Journal of Toxicology Environmental Health Part A*, 78: 945-957. DOI**:**10.1080/15287394.2015.1051206.

Chapter 4

Vaz-Velho, M., Silva, M., Pessoa, J. and Gibbs, P. 2006. Inactivation by Ozone of *Listeria innocua* on salmon-trout during cold-smoked processing. *Food Control,* 17: 609-616.

Silva, M. V., Ho, P. and Gibbs, P. A., 2019. Does a previous freezing step at -20ºC before product commercialization at chill conditions (5ºC) influence the microbial ecology of vacuum-packed cold-smoked salmon-trout (*Oncorhynchus mykiss*)? (*Submitted for publication in Journal of Food Science and Technology*).

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CHAPTER 2

Cold-smoked fish products: production, spoilage and microbiological contamination aspects

2.1. Introduction

Cold-smoked fish is characterized by specific chemical properties, based on lipid content 18% (w/w), water content <74%, an NaCl concentration of between 2.5 and 3.5% (w/w) and a smoke treatment corresponding to 0.6 mg of phenol per 100 g of product (Leroi, *et al*., 2000). These products are classified as lightly preserved fish products (LPFP), which include fish products preserved using low levels of salt (<6% NaCl (w/w) in water phase) and the addition of preservatives such as sorbate, benzoate, NO_2 or smoke for some products, having a high pH >5.0, packed in a vacuum or modified atmosphere and stored and distributed at temperatures of ≤ 5ºC (Lovdal, 2015). They are also classified as "ready-to-eat food" meaning "*food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level microorganisms of concern*" (Commission Regulation (EC) Nº 2073/2005 on microbiological criteria for foodstuffs). The same document specifies that 'products with pH ≤ 4.4 or aw ≤ 0.92, products with pH ≤5.0 and aw ≤ 0.94, products with a shelf-life of less than five days, are automatically considered as belonging to the category of RTE foods, but unable to support the growth of *L. monocytogenes*.

The microbial quality of the product depends on the spoilage potential of the microorganisms present and the storage conditions that affect the growth and formation of spoilage metabolites (Gram and Huss, 2000). Microbial control during processing and storage is a key factor that determines the quality and shelf-life of fresh fish. Bacteria on the product can originate from the raw materials or be introduced during processing by cross contamination from equipment or food handlers. Also, the salting/smoking processes are important processes, since they determine the physicochemical and microbiological characteristics of the end of smoked fish and their specific shelf-life.

The Atlantic salmon (*Salmo salar*) and salmon trout (*Oncorhynchus mykiss*) are the main raw materials used in cold-smoked fish industry. The preservative action of smoking is due to the combined effects of dehydration inherent to this drying process and the bactericidal properties of some chemicals present in smoke. Comparison of traditional smoke and liquid smoke on cold-smoked salmon trout were studied (Ayvaz and Atar, 2010; Ayvaz *et al*., 2017). Another complementary conservation action comes from salting, which is used along with smoking to different extents (Leroi and Joffraud, 2000). The temperature in cold smoking should be between 18ºC and 30ºC. The quality of the cold-smoked fish depends on the temperature to which it is subjected during drying, smoking and storage (Birkeland and Bjerkeng, 2005; FDA, 2001). The most common salting methods used in the manufacturing process are dry salting and brine or brine injection.

It has been suggested that intrinsic and extrinsic parameters such as the a_w, pH, and initial microbial load of a food product will determine the development of a specific characteristic in the product's microbiota (Huss, 1995). Studies have been published on the frequency, level, source and degree of contamination of cold-smoked fish and spoilage characteristics ("specific spoilage microorganisms") (Cardinali *et al*., 2004; Dondero *et al*., 2004) and on pathogenic microorganism identification, such as *L. monocytogenes* (EFSA, 2013). The origin of the fish and the freshness of raw material, aspects related to hygiene, environment plants (smoke factories) and the smoking process establish the selection of microorganisms and characteristics of the final products, with all these contributions affecting the presence of spoilage microorganisms and pathogens, such as *L. monocytogenes*.

This document presents a description of technological aspects of smoking process and the characteristics of associated curing methods in cold-smoked fish processing. Relevant aspects of microbiological contamination, spoilage and safety bacteria are described. Shelf life and factors related to quality of cold-smoked fish end products are also described.

2.2. Microbiological contamination

In cold-smoked fish processing, the fish habitat and processing environment are the main sources of microbial contamination of fresh fish and represent a major potential source of bacteria causing fresh fish to spoil. *L. monocytogenes* and *Clostridium botulinum* are the pathogenic microorganisms of major public health concern.

2.2.1 Spoilage microorganisms

The most commonly reported spoilage bacteria for aerobically stored chilled fish, including salmon, are species within the *Pseudomonas* (P.) and *Shewanella* (S.) genera, while the CO_2-resistant *Ph. phosphoreum* dominates in fish packed in a modified atmosphere (Chaillou *et al*., 2015; Dalgaard *et al*., 1993; Emborg *et al*., 2002; Parlapani and Boziaris, 2016; Tryfinopoulou *et al*., 2002). Strict hygiene during processing is a prerequisite for the optimal shelf life of salmon fillets and an approximate 90% reduction in the initial levels of bacteria on salmon fillets can be obtained by using optimal hygienic conditions (Moreto *et al*., 2016). As mentioned by the same authors, the processing environment in salmon processing plants represents a major potential source of bacteria that cause spoilage of fresh salmon: higher levels of *Pseudomonas* spp. and *Shewanella* spp. were found on fillets produced early on the day of production compared to fillets

processed later, while levels of *Photobacterium* spp. were not dependent on the processing method or time. *Ph. phosphoreum* produces trimethylamine (TMA) by reducing trimethylamine oxide (OTMA), a major spoilage product in fish (Dalgaard, 1995). The most important spoilage products of *Shewanella spp*. are volatile sulfides, but TMA may also be produced (Dalgaard, 1995; Joffraud *et al*., 2001). *Pseudomonas* spp. does not produce TMA but has been linked to changes in quality and the development of sweet, fruity off-odours in various species of chilled fish (Olafsdottir *et al*., 2006; Parlapani *et al*., 2015).

Temperature is very important in fresh fish storage, as both enzymatic and microbiological activities are temperature dependent. Many bacteria are unable to grow at a temperature below 10ºC and even psychotropic organisms grow slowly, and sometimes with extended lag phases, at temperatures approaching 0ºC. On fishing vessels and in processing industries, fish is contaminated by contact with surfaces, containers and utensils. The level of cleanliness has a significant influence on fish's bacterial load. Ice used to keep the fish cold may itself be contaminated and thus constitute an additional, not insignificant source of bacteria. Early evisceration is used to stop the action of digestive enzymes and the migration of intestinal bacteria to the muscle. However, when this process is carried out in poor hygiene conditions, intestinal microorganisms are spread on all the exposed surfaces of the fish. A good wash after evisceration makes it possible to considerably reduce this contamination (Moreto, 2016).

Superchilling is another technique that helps inhibit most autolytic and microbial reactions (Huss, 1995) and therefore extends the period of prime quality in fish. Several types of cooling systems (4ºC to 0ºC) have been used for the superchilling of seafood

products, including flake ice and slurry ice (Losada *et al*., 2006; Zeng *et al*., 2005) and subzero temperatures during storage (2ºC) (Sivertsvik *et al*., 2003).

After the smoking process, different bacteria, including LAB, *Brochothrix thermosphacta, Enterobacteriaceae, Aeromonas, Sh. putrefaciens* and marine vibrios, such as *Ph. phosphoreum*, and occasionally yeasts have been found during the storage of cold-smoked fish and were reported by various authors (Hansen *et al*., 1998; Leroi and Joffraud, 2011; Oguzhan and Angis, 2013; Silva and Gibbs, 2015). This microbial load does not necessarily coincide with the spoilage level and no relation has been found between the total number of microorganisms, sensory quality and shelf life (Hansen *et al*., 1996). Additionally, high LAB numbers (10^7-10^8 CFU/g) are often present for several weeks before the product is rejected sensorially (Dordevic *et al*., 2013). The development of active spoilage microbiota in smoked fish is not very well understood. A single compound quality indices has been appointed by some authors, as TMA levels (Leroi and Joffraud, 2000), the concentration of 2-Thiobarbituric Acid Reactive Substances (TBARS), pH (Espe *et al*., 2002), liquid–holding capacity (Rora *et al*., 2003; Hultmann *et al*., 2004) and biogenic amines (Jørgensen *et al*., 2000a, 2000b) have been studied to estimate the degree of spoilage for cold-smoked fish and fish products.

2.2.2 Pathogenic microorganisms

Pathogenic bacteria in cold-smoked fish can be divided into two groups (Huss *et al*., 1997; Huss *et al*., 1995):

1) Indigenous microbiota - which includes *C. botulinum*, *L. monocytogenes*, *Vibrio* spp. and *Aeromonas* spp. Most of these bacteria are found naturally in fish and thus in the final product.

2) Non-indigenous microbiota - this group comprises *Salmonella*, *Escherichia coli*, *Shigella* and *Staphylococcus aureus* which are not naturally present in fish and fish products

L. monocytogenes is a cold-tolerant facultative ubiquitous microorganism found commonly in soil, water and on plant matter. Ingestion of this microorganism may cause invasive listeriosis, resulting in meningitis, septicaemia, endocarditis, encephalitis, conjunctivitis and a flulike illness, often resulting in hospitalisation, (WHO, 2004). Although the incidence of listeriosis is relatively low (2,206 reported cases in the EU in the year 2015) (EFSA, 2016), the severe form of listeriosis is particularly dangerous to the immunocompromised and can have a mortality rate of up to 23.6% (DeNoordhout *et al*., 2014). Surveys and studies performed internationally have shown a wide prevalence of *L. monocytogenes* in cold-smoked salmon (no studies on hot smoked salmon specified), ranging from 0% (Pesavento *et al*., 2010) to 100% (Dauphin *et al*., 2001). However, after year 2000, the variation of *L. monocytogenes* in retail CSS was from 0 to 61%, with an average of 9.8% (Lovdal, 2015). The time and temperature used in the cold smoking process is favourable to the proliferation of different microbiota and harmful microorganisms (Hansen *et al*., 1995), such as *L. monocytogenes* that are present in all kinds of raw materials for food production. Lovdal (2015) reported that *L. monocytogenes* very rarely isolated from potable water and from fresh fish in pure water, meaning that it is introduced by contamination during processing. However, there is no step in the process that can fully ensure the absence of the pathogen. *L. monocytogenes* can grow on smoked

salmon under refrigeration temperatures (Guyer and Jemmi, 1991; Hudson and Mott, 1993; Hwang, 2007; Midelet-Bourdin *et al*., 2010), as well as proliferating at cold smoking chamber temperatures (Junttila *et al*., 1988; Seeliger and Jones, 1986). Therefore the major control measures against *L. monocytogenes* are the addition of salt and phenolic compounds (Cornu *et al*., 2006) and organic acids and carbonyls (Montazeri *et al*., 2013). Cold smoking processes do not offer sufficient listericidal capacities to render the product safe if initially contaminated (Hwang, 2007; Jemmi and Keusch, 1994). The processing environment itself has been shown to be a source of contamination (Chitlapilly-Dass *et al*., 2010; Dauphin *et al*., 2001; Di Pinto *et al*., 2010; Jordan *et al*., 2018; Vogel *et al.*, 2001).

C. botulinum is ubiquitous in nature and can be present in most types of raw foods (FDA, 2001a). In fish products, non-proteolytic *C. botulinum* that produces neurotoxin type E is the most important because it is found in most aquatic environments, both marine and freshwater (Gram, 2001b). A small number of studies have examined the prevalence of *C. botulinum* in cold-smoked fish, showing no or very little prevalence, and when present, it has a very small number of spores (Gram, 2001b). In cold-smoked fish products, the formulation and process are based on the need to inhibit *C. botulinum* spore germination, growth, and toxin formation. The interplay of the inhibitory effects of salt, temperature, smoke, and nitrite is complex. Control of the brining or dry salting process is important to ensure that there is enough salt in the finished product; however, preventing *C. botulinum* type E (and non-proteolytic types B and F) toxin production is made even more complex by the fact that adequate salt levels are often not achieved during brining. Therefore, proper drying is also important to achieve a finished product with the WPS level needed to inhibit the growth and toxin formation of *C. botulinum*. Control can be established by maintaining temperatures below 3.0-3.3^{o}C throughout distribution, retail and consumer storages, to inhibit the growth of all non-proteolytic and proteolytic strains (FDA, 2001a).

Maintaining temperatures consistently below 3.0-3.3°C, however, is not a realistic expectation based on current distribution, warehousing, retailing, and consumer handling practices. Consequently, a combination of both low temperature control and salt is vital. The inherent variability of WPS in this important process must be taken into consideration. This step is especially important in that WPS needs to be high enough to inhibit the outgrowth and toxin formation of *C. botulinum* in the final product. Brining should be done at a salt concentration that will provide an appropriate concentration in the final product (that is, 3.5% WPS in the final product). Acidity (pH), salt, moisture (and water activity) or combinations of these are performed to inhibit Clostridia outgrowth and toxin formation.

Although other human pathogens such as *Campylobacter jejuni* and *Yersinia enterocolitica* can be isolated from seafood, they are very sensitive to NaCl and are unlikely to grow in smoked products (Huss *et al*., 1995). If the refrigeration chain is maintained, the presence of *Bacillus cereus* and *Clostridium perfringens* can also be excluded as potential hazards because of the high temperatures they require to grow enough to produce toxins and their sensitivity to salt (Huss *et al*., 1995).

2.2.3 Parasites

Some of the fish species used for cold-smoked processing are intermediate or final hosts for parasites. For this reason, it is difficult to ensure the harvesting of parasite-free fish in the wild. Wild salmon can be infected with larval stages of the nematode *Anisakis simplex*. However, some fish with origins in aquaculture are considered to be free of parasites (if their feeding regime has not been supplemented with raw fish) because their diet can be controlled using net-pens, closed recycled systems or equivalents, and commercial pellet diets. Consequently, these control measures must be carefully

considered and applied, and no feed with raw fish must be introduced. Marty (2008) referred that *A. simplex* in the viscera as an estimate of its prevalence in muscle. The same author referred that the risk ratio of *Anisakid* parasites commercial products is 570 times less in farmed than in wild Atlantic salmon.

Nematodes do not survive at a temperature of 60ºC for 1 minute (FDA, 2001b). If a thread of 3 cm in thickness is subjected to 60 °C for 10 minutes, all parasites present in the product are killed (FDA, 2001b). In addition, dry salting does tend to kill parasites living on fish surfaces, but generally does not do so for those imbedded in the tissue. These results clearly demonstrate that WPS contents of 3-3.5% in cold-smoked fish would not be enough to kill the organisms. The Fish and Fishery Products Hazards and Controls Guide recommends a temperature below -20ºC for 7 d or -35ºC (internal) for 15 h to kill the parasites of concern (FDA, 2001b). It is therefore recommended that the cold smoking process should include a short period of freezing (-20ºC, 24 h) for fish, as a raw material or as a final product. Freezing raw fish prior to smoking remains the most effective way to ensure that viable parasites are not present in cold-smoked products consumed by the public (FDA, 2001b).

2.2.4 Active biogenic amine producers

Biogenic amines (BAs) are formed in foods as a result of microbial action during storage, usually during decomposition or spoilage processes involving the formation of free amino acids through proteolysis, together with bacteria production and the action of amino acids decarboxylases (Kuley *et al*., 2011). BAs are toxic substances and are blamed for producing diseases in humans and animals, especially with respect to histamine and tyramine (EFSA, 2011). Several Gram negative and Gram positive bacteria are able to

produce BAs. Spoilage bacteria belonging to enterobacteria and pseudomonads can accumulate histamine, putrescine and cadaverine (Linares, 2011), as some LAB species (Barbieri *et al*., 2019). The effect of specific LAB species on biogenic amine production by foodborne pathogen was reported by Ozugul (2011). BA content has been related to poor hygienic quality of non-fermented foods, being associated with a massive growth of decarboxylase positive spoilage microorganisms. The production of biogenic amines has been shown to be bacteria strain dependent (Garai *et al*., 2007).

BAs were studied as a single compound quality indices (Jørgensen *et al*., 2000a; Ozogul and Ozogul, 2006; Fadhlaoui-Zid *et al*., 2012). Predominant microbiota associated with cold-smoked fish including some *Enterobacteriaceae* and LAB are identified as active amine producers (Gram *et al*., 2002; Leroi *et al*., 2000; Silva et al., 2002). Jørgensen *et al*. (2000a) found *Ph. phosphoreum* to be primarily responsible for the production of biogenic amines in vacuum-packed cold-smoked salmon. More recently, a study developed by Silva and Gibbs (2015) emphasized the influence of storage temperature fluctuations on microbiological quality of cold-smoked fish samples and showed the presence of levels of LAB and the presence of active biogenic amine-producing bacteria, especially tyramine. Hansen (1995) referred that producers need be special attention to microbial contamination via poor hygiene conditions and fair manufacturing practices that might contribute to heavy spoilage and biogenic amines production. However, Huss *et al*., (1995) state that keeping the temperature below 5ºC from capture to consumption can eliminate the risk of histamine production. Considering cold-smoked salmon products, it is unlikely that histamine will become harmful to humans due to the critical concentration achieve (Jorgensen et al., 2000a; FDA, 2001a). The EU Regulation (EC) Nº 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs and EU Regulation (EC) Nº 1019/2013 of 23 October 2013 amending Annex I to Regulation (EC) Nº 2073/2005 as

regards histamine in fishery products. Histamine is the only biogenic amine with regulatory limits set by European Legislation, up to a maximum of 200 mg/kg in fresh fish and 400 mg/kg in fishery products treated by enzyme maturation in brine (Visciano *et al*., 2014). The amount of histamine in food, the individual sensitivity, and the detoxification activity in human organism represent the main factors affecting the toxicological response in consumers.

2.3. Smoking Process

Smoking is defined by its composition, and consists of a suspension of solid particles in a gaseous phase of air, carbon oxide, carbon dioxide, water vapour, methane, and other gases, making up an aerosol (Woods, 2003). The main factors affecting smoke composition are the type of wood, the temperature of combustion, the amount of water vapour available, the amount of oxygen present, the effect of air flow rate, and the smoking time (Woods, 2003). The temperature and constituents of wood are the most important parameters that affect smoking (Ahmad, 2003).

There are a number of ways of classifying smoking methods based on the temperature of the smoke, the location of smoke generation with respect to the position of the foodstuff, and the device used for generating smoke (Santiso *et al*., 2015). In cold smoking, temperatures must be kept below 30ºC throughout the process. Hot smoking should use temperatures between 70ºC and 80ºC (Santiso *et al*., 2015). The length of smoking is highly variable, from a few hours to even days in some processes. Hot or cold smoking can be used for the same species, depending on consumer market and the traditions in the region.

In direct smoking, smoke is produced in the same chamber where the product is processed. The traditional smoking method consists of direct thermal degradation of wood to produce smoke (Ahmad, 2003), like other kinds of smoking used in the past all over the world. Various unhealthy compounds are formed if the correct process is not used, especially the production of polycyclic aromatic hydrocarbons (PAH). EU Regulation (EC) nº 1881/2006 requires a formal setting new stricter rule on the content of PAH in smoked products.

Indirect smoking comprises a number of new methods that aim to reduce the formation of PAH contamination (Martinez *et al*., 2007b). Examples include smoke produced by a friction generator, liquid smoke and electrostatic smoking. Liquid smoke is a modern way of commercially smoking products more quickly and in a more environmentally sustainable (Montazeni, *et al*., 2013a). Liquid smoke is produced by condensing wood smoke formed in the controlled, minimal oxygen pyrolysis of wood, sawdust, or wood chips (Montazeni, *et al*., 2013a, 2013b). The gases produced are quickly chilled in condensers, thus liquefying the smoke. This is forced through refining vats and filtered to remove toxic and carcinogenic compounds. Liquid smoke exhibits antimicrobial activity against listeria (Martin *et al*., 2010), *Staphylococcus aureus* and their enterotoxins (Lingbeck *et al*., 2014).

The most common species of fish used in the smoking process include salmon, trout, herring, cod, mackerel, tuna, swordfish, oysters, and mussels.

2.3.1. Flues, wood and smoke

Flues are currently automated models that are able to control the temperature, humidity, ventilation and smoke flow in the smoking chamber. The process should ensure that the hygiene conditions of the smokehouse are checked and best practice procedures are followed.

The quality of the smoke depends on the type of wood used. The various types of wood used in smoking can be classified by structure, as hardwoods or softwoods. Hardwoods are traditionally used, especially oak and walnut, but also ash and beech. These types of wood are better because they provide a milder flavour in foods, compared to softwoods, which quickly have an impact on the colour of the product (Vaz Velho and Cappell, 1998). Treatments using commercial liquid smoke flavourings and two types of wood (beech and oak) on cold-smoked smoke generation were studied by Martinez *et al*. (2007a). These authors showed the different changes in physicochemical and sensorial attributes of cold-smoked salmon according to the different compositions of the liquid smoke flavouring, and a longer shelf life of CSS was observed in treatments similar to traditional smoking.

For proper cold smoke production, only chips or sawdust should be used. The wood should be burnt steadily, at low intensity, to be more controllable. It is important to ensure that the sawdust or chips are not dirty. Soil is very rich in fungi that can easily contaminate the product in the smoking stage (Madrid *et al*., 1994; Horner, 1997). Smoke is an emulsion of droplets formed by condensation in a continuous phase of air and steam, stabilized by electrostatic charges on the droplets. It comprises a particulate phase and a dispersed or gaseous phase (Vaz-Velho and Cappell, 1998). The particulate phase is the

phase that makes the smoke visible. The gaseous phase of the smoke is almost invisible and is formed by a huge number of compounds derived from the combustion of the wood.

The variety of compounds formed depends on the temperature at which combustion takes place. Wood smoke is composed of over 400 volatile components comprising 48 acids, 22 alcohols, 131 carbonyls, 22 esters, 46 furans, 16 lactones, 75 phenols and some 50 miscellaneous compounds (Ahmad, 2003; Woods, 2003, Santiso *et al*., 2015). The compounds are volatile in water, having properties similar to those of organic acids typically used as preservatives in food products, which diffuse to the muscle layers. Furthermore, water migrates to the surface of the fish and carried some proteins with it. After smoking, this film (protein and smoke) dries, forming a bright golden layer (Madrid *et al*., 1994; Horner, 1997). The bacteriostatic and antioxidant effects of smoke characterize the preservative action. During drying, the water activity of the fish decreases. Some components, such as thymol, formaldehyde, formic, acetic and benzoic acids, orthocresol, meta-cresol, para-cresol, guaiacol, methylguaiacol, cresol, and xinelone produce desirable antimicrobial and preservative effects (Lingbeck *et al*., 2014; Santiso *et al.*, 2015). Some phenolic compounds have antimicrobial activity against *L. monocytogenes*, however organic acids and carbonyls have been found to have more antilisterial and bacteriostatic properties than phenols (Montazeri *et al*., 2013; Milly *et al*., 2008). Also, antioxidants may be naturally present as the tocopherols induced by smoking (Vaz-Velho, 2003).

2.3.2 Cold-Smoking Process

As already mentioned, cold smoking occurs at temperatures below 30ºC (Ahmed, 1993). Humidity should be 60 to 70% or can be automatically controlled through the use of

wet sawdust (Madrid *et al*., 1994). The flue equipment is composed of a smoking chamber, where the fish is placed, and by a smoke-producing chamber, separated from the first, to which an air distribution system must be coupled. A water evaporation system is connected throughout the smoking process.

The flue should be prepared to perform the follow:

1) Dry air and smoke go through the fish at a variable speed between 0.2 and 0.5 m. s^{-1}. The smoke is dissolved on the wet surface of the fish and absorbed by the surface layer of the fillets, while the water on the fish's surface is lost through evaporation;

2) After contact with the fish, the air increases in humidity, so there should be a mechanism for cooling and to allow moisture loss. This mechanism is commonly a single heat exchanger with a valve to allow condensed water to be removed;

3) The air, which was cold and dry, is heated in a chamber where it produces smoke, and heated to the desired temperature, carrying a new amount of smoke;

4) The air, now hot and dry again, is brought to the smoking chamber, completing the air circulation cycle.

A controlled smoking process has a desirable effect on food colour, texture, smell and taste, and the various parameters of salting/drying/smoking have a major impact on the end product. Madrid *et al*. (1994) appointed the weight loss between 10-18 %. Birkeland and Bjerbeng (2005) showed that process yield was unaffected by temperature and decreased with salting time. The study also demonstrated that cold-smoked process is more important for variation in quality parameters than the salting process. Lerfall *et al*. (2011) concluded that smoking step is the major contributor to loss of astaxanthin in the fillet surface of cold-smoked Atlantic salmon (important to maintain the surface colour).

Figure 1 shows the flow scheme for the cold-smoked fish manufacturing process.

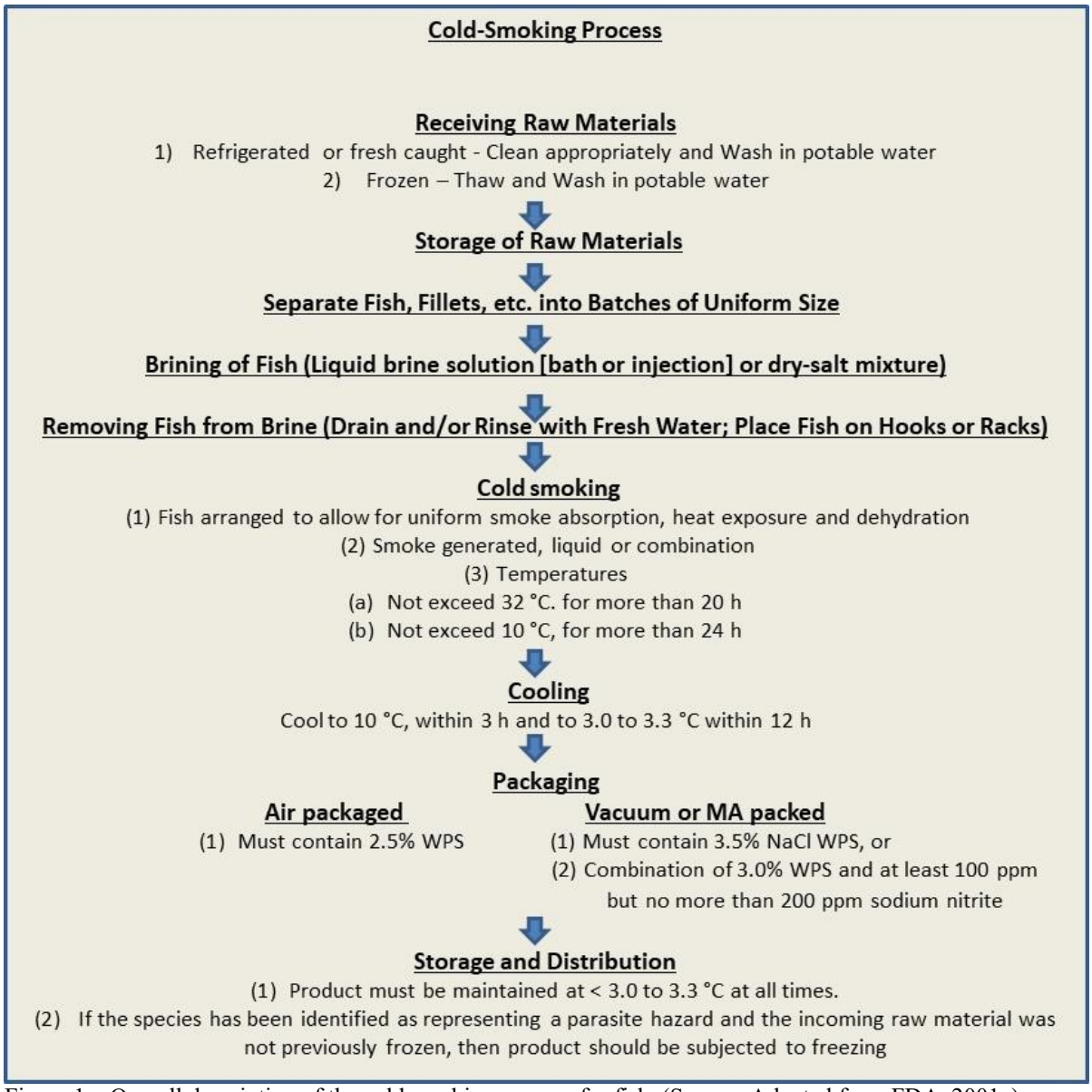

Figure 1 – Overall description of the cold smoking process for fish. (Source: Adapted from FDA, 2001a).

2.3.3 Raw fish and slaughtering

The characteristics of raw fish are determinant for quality of cold-smoked products. The protein content in salmon muscle is relatively constant, but may vary with season and fish size (Acharya, 2011). In wild salmon, higher levels of protein were found in the feeding season and less around the spawning season (Belitz *et al*. 2009). Fat content in

salmon fillets is essential for the texture, flavour and colour. The fat content in farmed adult salmon shows a high variation between and within the same population of fish (Mørkøre *et al*. 2001). Mørkøre & Rørvik (2001) reported that salmon accumulate substantial amounts of fat during the autumn, whereas the fillet fat content dropped slightly (by 1.5% units) during the winter. The effects on microstructure and texture of fresh and smoked Atlantic salmon (*Salmo salar*) under different slaughtered conditions were studied (Loge *et al*., 2007; Sigurgisladottir *et al*., 2001).

The fish used in the smoking process should be fresh or frozen. In both cases, it is important to check that suppliers' transport vehicles are kept cold. Rapid cooling before entering *rigor mortis* and maintaining a suitable appropriate temperature are the two main steps for preserving fish quality. Bjornevik *et al*. (2018) studied the effect of salting/smoking procedures on Atlantic salmon originating from *pre*-or *post rigor* filleted raw material on quality of end of product. Also freezing preserves quality allowing an expanded distribution range for raw fish, depending of different methods. Quality measures affected by freezing include changes in colour, texture, water holding capacity and intracellular/extracellular ice crystal growth effects on structure (Dawson *et al*., 2018).

The freshness of the fish gradually decreases after fishing due to two processes: physical and chemical changes resulting from bacterial growth and metabolism. EU Regulation (EC) Nº 2406/96 mentions a sensory method for seafood based on the EU quality grading scheme for fresh fish. EU Regulation (EC) Nº 790/2005 of 25 May 2005 amending UC (EC) Nº 2406/96. This method is to be used at the first point of sale and involves freshness and other quality aspects (parasites, pressure marks, injuries, blemishes and bad discolouration). The freshness rating uses a scale (Extra, A and B) in which the Extra, is the greater of freshness. Quality Index Method (QIM) is another scaling method

that establishes robust data reflecting the different quality levels of fish (Sant' Ana *et al*., 2011). QIM is based on significant sensory quality parameters for whole fish using many weighted quality parameters and a score system from 0 to 3 demerit points. In the QIM scheme for farmed salmon the quality parameters are skin, eyes, gills and abdomen. Each quality parameter is divided with a description of each (Hyldig and Green-Petersen, 2004). The scores for all the characteristics are added to give an overall sensory score, the so-called quality index (QI). A QI of zero is given for very fresh fish and QI score increases as the fish deteriorates. More recently, Garcia *et al*. (2017) proposed a smart quality sensor which enables to measure quality and to predict its progress through time. The sensor combines information of biochemical and microbial spoilage indexes with dynamic models to predict quality in terms of the QIM and EU grading criteria. Besides, the sensor can account for the variability inside the batch if spoilage indexes are measured in more than one fish sample. The sensor is designed and tested to measure quality in fresh cod (*Gadus morhua*) under commercial ice storage conditions. Only two spoilage indexes, psychrotrophic counts and total volatile base-nitrogen content, were required to get accurate estimations of the two usual established sensory methods.

Slaughtering fish must involve cleaning and washing procedures for the fresh or frozen fish. It is advisable to wash it using drinking water through a continuous flow water system. When frozen fish is used, thawing should be carried out slowly to 4ºC over 12-18h (Vaz-Velho and Capell, 1998). In all the processes, hygiene and sanitary norms must be applied in accordance with EU Regulation (EC) Nº 852/2004 on the hygiene of foodstuffs.

The size of whole fish or fillets are very important in the efficacy of the salting and smoking process because greater thickness makes the drying process and intake of smoke more difficult (Turan and Erkoyuncu, 2012). Large fish, such as swordfish or tuna, are

usually purchased frozen when already eviscerated and beheaded. They must be thawed slowly in the cold room or under cold running water. When more than 2/3 of the fish has thawed, an incision is made along the dorsal edge to begin to separate the two right and left large muscle masses.

Medium size fish includes fish such as trout, mackerel, herring or small cod. The fish are usually gutted and headless. Then the right and left threads are separated from the ventral edge, but held together by the dorsal edge. The spine is removed. They are then washed. At the end of the process, the skin may or may not be removed. Salmon and trout are prepared in the same way, but the left and right fillets, which are large, are completely separated from one another. The skin is usually removed at the end of smoking process.

2.3.4 Salting

Salting of fish is a process in which fishery products come into contact with salt in suitable containers or barrels, and consequently the product attains the desired structure and salinity. Adding salt has two main objectives: increasing flavour and preserving the fish. Salted fish can be preserved for a long time because water is discharged from the body of the fish by osmosis and the water content in the fish is reduced. At the beginning of the salting process, the outer salt concentration is higher, whereas the salt concentration in the fish is lower. Due to the concentration difference between the two environments, the osmotic pressure of the outer solution is higher than that of the fish, and thus salt intake and water discharge take place.

In order to obtain a high-quality product, the viscera are removed and, if necessary, the head is removed immediately after landing (Tunalı, 1984). Cleaning should be

performed very quickly, otherwise bacteria which have been transmitted to the fish from the seawater attack the tissues after the fish has died and lead to spoilage. The second purpose of cleaning is the easier and quicker penetration of saline water into the ventral cavity and inner sections of the fish (Turan and Erkoyuncu, 2012).

The intake of the salt into the fish tissue is dependent on the salt concentration available, temperature and texture (Knockaert, 1990). The main impurities that are found in the salt are calcium and magnesium salts. The salt used should contain 0.15-0.35% calcium and 0.15% magnesium and the amount of copper and iron should be between 0.1 ppm and 10 ppm respectively (Regenstein and Regenstein, 1991; Madrid *et al*., 1994).

Salting may be dry or wet. Fish salted with dry salt lose water due to the hygroscopic characteristics of salt and osmosis. The extracted water forms a concentrated salt solution together with the outer dry salt. Osmosis continues until the salt concentration inside the fish and the outer salt concentration equalize. In dry salting, salt penetration happens quickly, the a_w of the end product in brining is lower than in dry curing (Knockaert, 1990).

A salt concentration of 5 to 10% (W/W) completely covers the surface of the fish. Sugar can also be added, which gives a particular flavour and helps reduce the salty taste. Excess salt is removed using water. The curing time depends on the size and presentation of the fish (whole, fillet, with or without skin). Salting must be at a temperature of 12-15°C (Knockaert, 1990). The size of the salt crystals is important in salting. In general, fine salt is advantageous because it spreads over the whole fish more uniformly. However, since fine salt rapidly absorbs water from surface tissue within the first days of salting, it coagulates protein and hardens the tissue. As a result, it may lead to spoilage because of slower salt intake. In the tissues which are closer to the surface, proteins can precipitate.

Wet curing is usually carried out using 70-80º brine, which may or may not be enriched with sugar and spices. A saturated brine may affect the end product due to the possibility of salt crystals forming on the surface (Vaz-Velho and Cappell, 1998). The solubility of salt in water is 35.8g/100g of water at 16ºC (Turan and Erkoyuncu, 2012). Accordingly, a saturated salt solution contains 26.4% (35.8 *100/135.8) salt. The solubility of salt change slightly with the temperature (Turan and Erkoyuncu, 2012). Brine contains 9–11% salt in light salting, 14–16% salt in medium salting, and 24% salt in heavy salting (Turan and Erkoyuncu, 2012). The brine preserves its concentration, continuously absorbs water from the fish, and the exchange continues as long as salt crystals are present in the solution. As soon as the salt in the fish reaches 9–10%, irreversible changes take place in the proteins, causing denaturation (Turan and Erkoyuncu, 2012).

A good quality brine should be clear, transparent, without much foam and without an unpleasant odour, and it must be renewed frequently to avoid contamination of the product with scales, entrails, debris and lumps formed by the fish protein dissolved in water (Turan and Erkoyuncu, 2012). The appropriate concentration of the brine depends on the type of product and the brine step period. In both salting methods (dry and wet salting), the water content rate of the fish decreases because the water in the fish is discharged. The decrease is higher in the dry salting method (Cardinali *et al*., 2001). Gallart-Jornet *et al*. (2007b) referred that the yield after salting was higher when more diluted brines were used, as compared to saturated brine and dry salting.

2.3.5 Drying

After salting, the fish undergo “dripping” or drying. This consists of remaining in a cold chamber for a time at a temperature between 4ºC and 6°C (Ahmed, 1993). The purpose of this operation is not exactly to dry the fish but to encourage the salt present, especially concentrated on the surface layer of the threads, to migrate slowly to the deeper layers, and consequently affect the water loss. In general, for small fish, an overnight stay in the cold room is enough, while 48 hours, or more would be necessary for larger fish.

2.3.6 Packaging and storage conditions

Typically, the cold smoked fish is presented in the form of fillets, cubes or slices vacuum-packed in polyethylene bags and stored in refrigerated conditions (4 to 6°C) or frozen (FDA, 2001a). Usually, the products are commercialized without the skin. These commercial presentations still very similar nowadays but sometimes with a new look in packaging.

Smoked fish may also be packaged in a modified atmosphere. MA involves removing air from the package and replacing it with a single gas or mixture of gases to enhance the food’s shelf life. The gaseous atmosphere inside a MA pack changes continuously during storage due to the absorption of gases by the foods, respiration of certain products, microbial growth and the exchange of gases through the package (Ooraikul, 2003). Modification of the atmosphere in a package involves a reduction in oxygen or an increase in carbon dioxide/nitrogen concentration, but in some cases an amount of carbon monoxide, ethylene, ethanol or other compounds in the atmosphere can

also be used to extend shelf life (Erkmen, 2012). It is recommended for the gas mixture to contain 60% carbon dioxide and 40% nitrogen (FDA, 2001a).

Microorganisms differ in the gaseous atmospheres they require for growth (Day, 2008; Erkmen and Bozoglu, 2008b) as follows: 1) aerobic microorganisms require oxygen to grow, such as *Pseudomonas*, *Psychrobacter*, *Shewanella*, *Acinetobacter/Moraxella*, *Micrococcus*, some species of *Bacillus*, film yeasts, and moulds. Growth inhibition of these microorganisms can be achieved by excluding oxygen from the MA; 2) Microaerophilic microorganisms require low levels of oxygen for growth. Some may require increased levels of carbon dioxide for growth, such as *Campylobacter* and *Lactobacillus*; 3) Facultative anaerobic microorganisms are able to grow in the presence or absence of oxygen, such as *Escherichia coli*, *Staphylococcus aureus*, *L. monocytogenes*, *Brochothrix*, *Salmonella*, *Vibrio*, *Aeromonas*, some species of *Bacillus*, lactobacilli, and fermentative yeasts; 4) Anaerobic microorganisms are inhibited or killed by oxygen, such as *Clostridium* and *Bifidobacterium.*

Some studies have shown that a single antibacterial treatment may not be adequate to inhibit pathogenics microorganisms in fish products, therefore the hurdle technology has been employed to control growth of pathogens in products, and retain quality (Tocmo, 2014). The concept of combined preservative factors may improve microbiological and sensory quality in foods as a result of synergistic effects of combined preservatives factors (Leistner, 2000).

2.4. Shelf life and cold-smoked fish quality

Cold-smoked fish are often packaged in vacuum having a limited shelf life, even at refrigeration temperature. They and must be stored and distributed at refrigeration ≤ (5ºC) or frozen temperatures. According to Huss *et al*. (1995) three weeks at 5 ºC should be the maximum storage for vacuum packed cold-smoked salmon. However, FDA (2001a) reported that the shelf life of such products depends of the salt content applied in the smoking process, precisely the WPS of end of product and, the atmosphere of packaging and temperature conditions, resulting In general, the shelf-life is indicated between 3 to 5 weeks. The Table 1. summarize the quality characteristics and shelf-life time of cold-smoked fish products obtained in different research studies.

The shelf-life reduction of fishery products is largely due to enzymatic activity (Laksmanan *et al*. 2003) and microbial activity (Bugueno *et al*., 2003). As observer before, the absence of thermal treatment, the parameters of salting and drying/smoking are crucial steps to achieve the ideal WPS level in order to minimize the risk of foodborne hazards and spoilage (Lovdal, 2015). The shelf life based on sensory evaluation, range from 3 to 6 week at 5^{o}C (Rorvik, 1991). According to current United States Hazard Analysis Critical Control Point (HACCP) regulations, a suggested critical limit for air-packaged product is at least 2.5% NaCl (water phase in the loin muscle), for vacuum-packaged or modified atmosphere-packaged product at least 3.5% NaCl (water phase in the loin muscle), or a combination of 3.0% WPS and at least 100, but not more than 200 ppm, of sodium nitrite (allowed in the United States for sable, salmon, shad, and chub).

Table 1. – Studies on quality and shelf-life obtained for chilled cold-smoked fish products

Fish species	Salting/Smoking process	Shelf life (days or weeks)	Quality based on sensory, physicochemical or microbiological characteristics	Reference
Atlantic salmon (*Salmo salar*)	Salting/Ripening; Cold-Smoking (20-28ºC)	2-3 w (3-4% WPS)	Microbiological characteristics	Hansen et al. (1995)
Atlantic salmon (*Salmo salar*)	Injection brining (23-25%) and Drysalting; Cold-Smoking (20-26ºC)	21-36 d for sliced and 32-49 d for fillets at 5ºC (3-7w)	Sensory characteristics	Hansen et al. (1998)
Atlantic salmon (*Salmo salar*)	Brining/Drysalting and cold-smoking (20ºC and 30ºC) by electrostatic and traditional smoking	nd	Physical characteristics	Sigurgisladottir et al. (2000)
Atlantic salmon (*Salmo salar*)	Drysalting and Traditional cold-smoking (20-26ºC)	1-6 w (or more) at 5ºC	Physicochemical (TMA, TBVBN, ..), microbiological and sensorial characteristics	Leroi et al. (2001)
Atlantic salmon (*Salmo salar*)	Dried/Drysalted (2,7%NaCl); Cold-Smoking (24ºC)	7-26 days at 5ºC (1-4w)	Sensory, chemical (TMA, TVB, Kvalue) and microbiological characteristics (TVC, Anaerobic Counts and *Lactobacillus* spp.)	Dondero et al., (2004)
Atlantic salmon (*Salmo salar*)	Liquid smoke flavourings	21% of samples, 2w at 4ºC	Physicochemical and microbiological characteristics	Cardinali et al. (2004)
Atlantic salmon (*Salmo salar*)	Drysalting or injection salting/extended drysalting; Cold-smoked	nd	Physicochemical characteristics of raw salmon (carotenoid composition and fat content) and end product (Yield, phenols, colour, texture, LHC, gaping)	Birkeland et al. (2004)
Atlantic salmon (*Salmo salar*)	Brine (saturated or 50%) and drysalting	nd	Physical characteristics (carotenoids retention, salt content and yield)	Birkeland and Bjerkeng (2005)
Atlantic salmon (*Salmo salar*)	Liquid smoke flavourings	19-45 days at 5ºC (2-6w)	Sensory and physicochemical characteristics	Martinez et al. (2007)
Atlantic salmon (*Salmo salar*)	Brine salted	nd	Textural properties	Indrasena et al. (2008)
Atlantic salmon (*Salmo salar*)	Drysalting or injection salting	nd	Physical characteristics (carotenoids retention)	Birkeland and Bjerkeng (2011)
Atlantic salmon (*Salmo salar*)	Liquid smoke flavourings	34-45 days at 5ºC (5-6w)	Sensory, physicochemical and textural characteristics	Martinez et al. (2012)
Atlantic salmon (*Salmo salar*) Salmon trout (*Onchorrynchus mykiss*)	Traditional cold-smoking processing	2-6 w at 5ºC (depending of producers)	Microbiological characteristics	Silva and Gibbs (2015)
Rainbow trout (*Onchorrynchus mykiss*)	Unknown	60d (8w)	Microbiological and chemical characteristics	Iacumin et al. (2017)
Atlantic salmon (*Salmo salar*)	Dry salting or injection brine (salting targets, 2.5 and 4%)	nd (studies 4ºC during 4w)	Yield and quality (used pre-post filleting time)	Bjornevik et al. (2018)

Nd- Not determined

The intrinsic qualities of fish are not immutable characteristics. The characteristics of the fish after leaving their natural habitat can be maintained or lost. The deterioration of

the quality of the fish is essentially influenced by the factors time and temperature. The monitoring and control of temperature, from fishing to the consumer is the most important aspect of quality assurance, whether health, nutritional, organoleptic, texture were relevant.

Control of food safety and quality has been the subject of increase attention and the growing concern of consumers in relation to the matter of *L. monocytogenes*. The application of HACCP is recommended and widely applied in the establishments processing and preparing food.

CHAPTER 3

Microbiological and chemical characteristics of vacuum-packed cold-smoked salmon-trout during chill storage

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Production of histamine and tyramine by bacteria isolated from Portuguese vacuum-packed cold-smoked fish

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Abstract

An agar medium containing histidine or tyrosine incubated anaerobically was used for detecting the decarboxylating activity of bacteria isolated from Portuguese vacuum-packed cold-smoked fish during chilled storage. The capacity of each bacterial isolate to produce histamine and tyramine was studied at 25 and 5 °C incubated for 48 h and 10 days, respectively. More strains produced histamine and tyramine at 25 °C compared with 5 °C although lactic acid bacteria (LAB) strains exhibited similar results at the two different temperatures. Tyramine was produced by majority of the isolates tested although very low concentrations were produced at 5 °C as confirmed by high-pressure liquid chromatography (HPLC). Tyrosine-agar was shown to be a good indicator medium for detection of bacteria that produced high levels of tyramine, since typical colonies surrounded by a translucent halo were easily recognised. LAB identified as *Lc. Lactis lactis* 1 and *Carnobacterium divergens* were detected as tyramine-producing bacteria. *Acinetobacter* spp. and *Pseudomonas* spp., isolated from all Portuguese smoked fish products, were negative on histidine-agar, but HPLC identified considerable quantities of histamine produced in a broth medium.

Keywords: Histamine; Tyramine; Biogenic amines; Decarboxylating bacteria; Cold-smoked fish

1. Introduction

Many authors have discussed the toxicology of biogenic amines and their occurrence and formation in foods, with special emphasis on fermented foods. Several biogenic amines play important roles in many human and animal physiological functions (ten Brink, Damink, Joosten, & Huis in't Veld, 1990) but the consumption of high amounts of these amines can have toxicological effects. The most notorious food-borne intoxications caused by biogenic amines are related to histamine, referred to as "scombroid fish poisoning". On the other hand, the toxicology of tyramine is usually associated with cheese consumption.

Furthermore, biogenic amines have been used as chemical indicators of seafood quality especially on vacuum-packed cold-smoked salmon (Jorgensen, Huss, & Dalgaard, 2000a). The same authors showed four different biogenic amine profiles at the time of spoilage in cold-smoked salmon, and these were dependent on the composition of the spoilage microflora. Research on identification of spoilage microflora and biogenic amine production of single and co-cultures growing in cold-smoked salmon was also studied by Jorgensen, Dalgaard, and Huss (2000b). Strains of Enterobacteriaceae and lactic acid bacteria (LAB) are the main microflora associated with cold-smoked fish and identified as active amine producers (Joosten & Northolt, 1989; Jorgensen et al., 2000a; Leisner, Millan, Huss, & Larsen, 1994).

Truelstrup Hansen (1995) described three scenarios that characterise the spoilage microflora in cold-smoked salmon: (i) LAB, (ii) dominance of a mixture of LAB and Enterobacteriaceae and (iii) dominance by *Photobacterium phosphoreum* or other marine members of the Vibrionaceae family, often together with LAB. With respect to biogenic amine production, Jorgensen et al. (2000b)

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showed that *P. phosphoreum* was the only species that produced histamine when inoculated on sterile cold-smoked salmon and the production of putrescine was increased when cultures of *Serratia liquefaciens* or *Hafnia alvei* were grown with *Carnobacterium divergens* or *Lactobacillus sakei subs. carnosus*.

Biogenic amines in foods are usually generated by microbial decarboxylation of amino acids present in muscle tissues. Several factors (the incubation temperature, the pH and the growth medium composition) are known to affect bacterial growth and decarboxylase activity. Differential agar media were used in various previous studies on selection or on recognition of decarboxylating bacteria from fish (Chen, Wei, Koburger, & Marshall, 1989; Leisner et al., 1994; Niven, Jeffrey, & Corlett, 1981) and other food products (Actis, Smoot, Barancin, & Findlay, 1999; Joosten & Northolt, 1989; Maijala, 1993).

Based on high detection rates (93.9%) obtained using Niven's medium (Niven et al., 1981) to select histamine-producing bacteria (Chen et al., 1989), this medium was selected in this study as a differential agar medium to detect histamine- and tyramine-producing bacteria that have been isolated from Portuguese cold-smoked fish. Also, a modified decarboxylation agar proposed by Maijala (1993) was included in this study for detection of the same amines produced by LAB. Histamine and tyramine are reported as the more important biogenic amines that contribute to spoilage on cold-smoked salmon and are produced especially by *P. phosphoreum* and *Carnobacterium piscicola*, respectively (Jorgensen et al., 2000a). The purpose of this investigation was to study the production of amines by bacterial strains that formed the dominant microflora on Portuguese vacuum-packed cold-smoked fish stored at 5 °C, using decarboxylation agars and confirmation by high-pressure liquid chromatography (HPLC).

2. Materials and methods

2.1. Cold-smoked fish samples

Vacuum-packed cold-smoked fish samples stored at 5 °C were obtained from three different Portuguese smokehouses, during week storage.

2.2. Microbiological analysis

Vacuum packs of cold-smoked fish were stored at 5 °C for up to 5 weeks. At weekly intervals, a pack from each smokehouse was opened and 30 g of cold-smoked fish was taken aseptically (10 + 10 + 10 g, from different sites) and homogenised for 90 s in a stomacher. 10 g of this mixture was aseptically taken and decimally diluted in sterile maximum recovery diluent (CM 733 Oxoid) and homogenised for 20 s. Total viable counts were performed on spread plates of Long and Hammer's medium (LH) modified by Van Spreekens (1974) (with additional 1% w/v NaCl) incubated aerobically at 21 °C for 3 days. Counts of LAB were made on pour plates of nitrite-actidione-polymyxin (NAP) agar pH 6.7 (Davidson & Cronin, 1973) incubated anaerobically (Anaerocult A, Merck) at 21 °C for 5 days. Enterobacteriaceae counts were made by pour plates of 5 ml tryptone soya agar (TSA) (Lab M), which after incubation at 20–25 °C for 2 h were overlaid by 12–15 ml of violet red bile glucose agar (Merck) (TSA/VRBGA). Typical Enterobacteriaceae colonies were counted after 2 days incubation at 30 °C.

3. Bacterial isolates

Representative colonies that grew on Long and Hammer's plates were picked and the following tests were performed: cell morphology, Gram stain, catalase and oxidase tests. The cultures were maintained on nutrient agar slants and stored at 5 °C. Identification of the LAB group was based on the Gram reaction, absence of catalase and cytochrome oxidase and fermentative catabolism of glucose. For selected strains API 20N (BioMérieux) and API 50CHL (BioMérieux) were used for identification of Gram negative strains and LAB, respectively.

A strain of *C. divergens* previously isolated from cold-smoked salmon and obtained from the culture collection of ENITIAA (Nantes) was included in this study as a reference.

4. Examination of amine-forming capacity

Thirty one Gram-negative and 17 strains that were included in the LAB group were selected to investigate the decarboxylating capacity.

Media used to detect decarboxylating strains was prepared as described by Niven et al. (1981) (0.5% tryptone (Lab M), 0.5% yeast extract (Lab M), 2.0% L-histidine-monohydrochloride (Oxoid) or L-tyrosine (Oxoid), 0.5% NaCl (Merck), 0.1% $CaCO_3$ (Merck), 2.0% agar (Lab M) and 0.006% bromocresol purple, at pH 5.3). Additionally, the decarboxylation agar proposed by Maijala (1993), a modified version of the medium described by Joosten and Northolt (1989), was also used to detect decarboxylating LAB strains (0.5% tryptone (Lab M), 0.8% Lab-Lemco powder (Oxoid), 0.4% yeast extract (Lab M), 2.0% L-histidine-monohydrochloride (Oxoid) or L-tyrosine (Oxoid), 0.05% Tween 80 (Merck), 0.02% $MgSO_4$ (Merck), 0.005% $MnSO_4$ (Merck), 0.004% $FeSO_4$ (Merck), 0.01% $CaCO_3$ (Merck), 2% agar (Lab M) and 0.006% bromocresol

purple, at pH 5.3). Both media were autoclaved for 10 min at 121 °C to avoid excessive hydrolysis of the agar at low pH.

Strains were prepared for testing by subculturing in nutrient broth (NB) (Lab M) supplemented with 0.4% each of the amino acids histidine or tyrosine and incubated at 25 °C for 48 h. A loop of each culture was spread on the decarboxylation agars which were then incubated anaerobically (Anaerocult A, Merck) at 25 and 5 °C for 48 h and 10 days, respectively. A purple halo was interpreted as positive for amine production on both media with the exception of the decarboxylation media containing tyrosine that also produced a clear halo surrounding the colonies for a positive reaction, as proposed by Joosten and Northolt (1989).

All the strains were tested twice on separate occasions.

4.1. Analysis of broth culture media for amines

For selected strains that were on dominance on cold-smoked fish samples, a pre-culture were prepared as described above and 100 μl of each pre-culture was inoculated into 5 ml of NB (Lab M) supplemented with 0.4% of histidine or tyrosine and incubated at 5 °C for 25 days.

One ml of the culture fluid was mixed with 1 ml of hydrochloride acid solution (0.1 M) (Fluka), homogenised and kept in an ultrasonic bath for 15 min and then filtered through a 0.22 μm membrane (Technokroma). The filtrate was centrifuged for 5 min and 30 μl of the filtrate was derivatised using dabsyl chloride at 70 °C for 15 min. The reaction was stopped by placing the vials in an ice bath for 5 min. The dabsylated derivates were diluted in a mixture of acetonitrile and ethanol and after centrifugation, separation and quantification of tyramine and histamine were performed by using HPLC.

The chromatographic analyses were carried out in a JASCO high performance liquid chromatograph equipped with a JASCO PU-1580 pump, a JASCO LG-1580-04 quaternary gradient unit, a type 7125 Rheodyne Injector with a 20 μl loop, a Gilson model (temperature control module) oven and with a JASCO UV-970 UV/Vis detector. The integrator used was a Borwin PDA controller software.

The separations were performed at 50 °C on a 150×4 mm^2 I. D. Spherisorb ODS C 18 column, 3 μm. Dabsylated amines were eluted at a flow-rate of 1 ml/min using a gradient made with mobile phase (A), consisting of 9 mM sodium dihydrogen-*ortho*-phosphate, 4% dimethylformamide and 0.1% triethylamine (TEA), titrated to pH 6.55 with phosphoric acid and mobile phase (B) was 80% (v/v) aqueous acetonitrile (Krause, Bockhardt, Neckermann, Henle, & Klostermeyer, 1995). Detection was at 436 nm. Quantification was carried out using histamine and tyramine standards.

5. Results and discussion

LAB were determined as the dominant group present in Portuguese vacuum-pack cold-smoked fish and is in agreement with other previous studies (Jorgensen et al., 2000b; Leisner et al., 1994; Truelstrup Hansen, 1995). There were some differences in the development of LAB and Enterobacteriaceae in products from the three smokehouses (data not shown).

From the studies using the agar media, strains of decarboxylating bacteria were identified based on the appearance of a purple colour around the colonies (Table 1). Niven's agar identified 26% and 13% of histamine-producing Gram-negative bacteria at 25 and 5 °C, respectively (Table 1). Most of these bacteria belong to the genera *Serratia* and *Enterobacter* (results not shown) and these seem to be the most frequently isolated Enterobacteriaceae from cold-smoked salmon (Jorgensen et al., 2000a; Truelstrup Hansen & Huss, 1995). Niven's agar was used in previous studies and showed a high detection rate of histamine-producing bacteria (Chen et al., 1989; Joosten & Northolt, 1989). In the present study, additional confirmation of histamine and tyramine production by HPLC was studied on selected bacterial strains (Table 2). One false positive and two false negative results for histamine detection were observed for bacterial strains tested on agar media. In fact the agar media test is based on the production of an alkaline reaction and not on the specific detection of histamine. The production of ammonia and not histamine can be a reason for false positive detection on agar (Actis et al., 1999; Jorgensen et al., 2000a). In contrast, false negative reactions can occur on differential plating media based on excessive acid production that consequently neutralises the increase of pH resulting from amine production. Actis et al. (1999) observed that pH was increased by about two units by histamine-producing bacteria but was drastically reduced when the histidine-containing medium was supplemented with peptone, beef extract and glucose. In the current study, *Pseudomonas* spp. and *Acinetobacter* spp. were identified as two strong histamine producers that did not show a positive reaction on Niven's agar. These particular bacteria are not very common on cold-smoked salmon but in fact were isolated from products from all smokehouses. In contrast, bacteria commonly isolated from these type of products such as *P. phosphoreum* (Jorgensen et al., 2000b) showed that only 6% of these strains produced an alkaline reaction from histidine in Falkow's medium. Also, *Hafnia alvei* failed to show a positive reaction colour change in Niven's medium (Chen et al., 1989).

Table 1
Number of positive histamine and tyramine-producing bacteria determined using decarboxylation agars

	Decarboxylation agar (Niven et al., 1981)				Decarboxylation agar (Maijala, 1993)	
	At 25 °C, 48 h		At 5 °C, 10 days		At 5 °C, 10 days	
	Histamine	Tyramine	Histamine	Tyramine	Histamine	Tyramine
Gram negative						
31	8/31	25/31[a]	4/31	19/31[b]	nd	nd
Gram positive						
LAB 17	0/17	4/17	0/17	4/17	0/5	6/17
Others 5	0/5	1/5	1/5	1/5	1/5	2/5

nd: not determined.
[a] Positive reaction based on a visible clear halo around the colonies.
[b] Weak positive reaction based on the appearance of purple colour around the colonies.

Table 2
Production of histamine and tyramine by selected bacterial strains isolated from Portuguese cold-smoked fish studied with HPLC method

Strain designation	Source	Storage week	Supplemented in NB (25 days at 5 °C)		Bacterial identification
			Histamine (mg/l)	Tyramine (mg/l)	
					API 20E
4	Plant C (salmon)	1	0.00 (n)	1.30 ± 0.04 (w)	*Aeromonas* spp.
14	Plant B (salmon)	3	0.00 (w)	3.20 ± 0.13 (w)	*S. liquefaciens*/*S. marcescens*
63	Plant C (salmon)	2	73.7 ± 0.85 (n)	11.71 ± 0.40 (w)	*Pseudomonas* spp.
70	Plant C (salmon)	3	0.00 (n)	7.27 ± 0.20 (w)	*Pseudomonas* spp.
72	Plant B (salmon)	3	0.00 (n)	8.06 ± 0.08 (n)[a]	*P. fluorencens*/*putida*
73	Plant A (salmon)	1	341.8 ± 1.84 (n)	4.08 ± 0.03 (w)	*Acinetobacter* spp.
74	Plant A (salmon)	1	21.34 ± 2.19 (w)	5.90 ± 0.62 (w)	Unknown
C. divergens	Culture collection		0.00 (n)	8.25 ± 0.10 (p)	
			Supplemented in NB (48 h at 25 °C)		
LAB					API 50CHL
34	Plant C (salmon-trout)	1	0.00 (n)	453.6 ± 0.92 (p)[b]	*C. divergens*
35	Plant C (salmon-trout)	1	nd (n)	nd (n)[b]	*Lc. Lactis lactis* 1
37	Plant B (salmon)	3	nd (n)	nd (n)[b]	Unknown
41	Plant A (salmon)	3	0.00 (n)	646.5 ± 1.98 (p)[b]	*Lc. Lactis lactis* 1

nd: not determined; (p), (w) and (n): positive, weak positive and negative reaction, respectively, on decarboxylation agar (Niven et al., 1981).
[a] Development of weak yellow colour on Niven's agar.
[b] Positive reaction on decarboxylation agar proposed by Maijala (1993).

When this method was used for screening of histamine-producing bacteria some histidine decarboxylase-producing bacteria may be excluded at the low pH of the medium (Chen et al., 1989).

A high detection rate using Niven's medium was observed especially when high levels of tyramine were produced at 25 °C (Table 1). The Gram-negative strains in general produced tyramine but low levels were found at 5 °C (Table 2). Confirmation by HPLC indicated one false negative bacterium identified as *P. fluorescens*/*putida*, probably due to the excessive acid production that contributed to mask the purple colour on agar plates.

On the other hand, two strong tyramine-producing LAB strains that were identified as *Lc. lactis lactis* 1 and *C. divergens* were quantified (Table 2). The first bacterium used as a commercial starter culture has already been commented upon as a tyramine producer (Maijala, 1993). This author showed that the recognition was based on a HPLC method and not on using decarboxylation agar, even the modified decarboxylation agar for LAB strains. In our study, 35% of the LAB strains were detected as tyramine producers on the modified decarboxylation agar (Maijala, 1993) and of these only 24% of positive strains were detected by Niven's medium (Table 1). The basal composition of the modified decarboxylation agar was given by Niven et al. (1981), but to enhance the growth of LAB strains some metal sulphates and Tween 80 were added. *C. divergens* was identified as very common on cold-smoked fish and is part of the spoilage microflora. Jorgensen et al. (2000a) reported that when the end of shelf-life was based on the Multiple Compound Quality Index (MCQI) for cold-smoked salmon, the minimal spoilage levels of *C. divergens* would be 10^9 cfu g^{-1}. The same authors showed that *C. divergens* and *Lactobacillus sakei* subsp. *carnosus*

enhanced the spoilage activity of *S. liquefaciens* or *H. alvei* but this was not shown in the case of *P. phosphoreum*. The inclusion of the term "metabiotic spoilage association" for situations where two or more species exchange metabolites, which results in spoilage, was introduced by Jorgensen et al. (2000b). In the current study, bacteria having amine-producing capacity were only studied individually and no studies using mixture cultures (*cocktail*) were made.

6. Conclusions

Tyrosine-agar was shown to be a good indicator medium for detection of bacteria that produce high levels of tyramine, since typical colonies surrounded by a translucent halo were easily distinguished. Also, a modified decarboxylation agar proposed by Maijala (1993) was shown to be more promissory for use on detection of tyramine LAB strains, probably due to the medium composition.

From the results obtained using Niven's medium, and especially for detection of histamine, considering that by-products such as ammonia can deliver false positive results, further research is needed to increase the selectivity of these differential media and identify the cause of the false negative results obtained with the genera *Acinetobacter* and *Pseudomonas*.

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SIGNIFICANCE OF BIOGENIC AMINES IN COLD-SMOKED FISH AND THEIR RELATION TO MICROBIOLOGICAL CHARACTERISTICS OF PRODUCTS AVAILABLE IN PORTUGUESE RETAIL MARKETS

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Studies on microbial characterization of cold-smoked salmon and salmon trout during cold storage were performed on samples available in the Portuguese market. Samples were also classified microbiologically according to guidelines for ready-to-eat (RTE) products. Further investigations on sample variability and microbial abilities to produce tyramine and histamine were also performed. The coefficient of variation for viable counts of different groups of microorganisms of samples collected at retail market point was high in the first 2 wk of storage, mainly in the Enterobacteriaceae group and aerobic plate count (APC), suggesting that microbiological characteristics of samples were different in numbers, even within the same batch from the same producer. This variation seemed to be decreased when storage and temperature were controlled under lab conditions. The numbers of Enterobacteriaceae were influenced by storage temperature, as indicated by low microbial numbers in samples from controlled refrigeration. Lactic acid bacteria (LAB) and Enterobacteriaceae were predominant in commercial products, a significant percentage of which were tyramine and less histamine producers. These results might be influenced by (1) the technological processes in the early stages of production, (2) contamination during the smoking process, and (3) conditions and temperature fluctuations during cold storage at retail market point of sale.

Seafood-borne diseases are a major concern of consumers, producers, and authorities and may be produced by a variety of agents, including aquatic toxins, biogenic amines, bacteria, viruses and parasites (Gram & Huss, 2000; Iwamoto et al., 2011). The growing consumer interest in food quality and nutritional issues, have contributed to the increase of consumption of fish and fish products. Risk perception studies showed that individuals may underestimate significant risks while overestimating others, lowering their perceived risk but not the actual risk. Despite the potentially high biological activity of many biogenic amines and their occurrence in several foods, these substances may be significantly underestimated (Burger et al., 1993; Burger, 1998; Kramer and Scott, 2004; Fatimah et al., 2011). High levels of biogenic amines may be formed before foods appear spoiled or organoleptically unacceptable. Biogenic amines are formed in foods as a result of microbial action during storage, usually formed during decomposition or spoilage processes involving formation of free amino acids through proteolysis, together with bacterial production and action of amino acids decarboxylases (Ten Brink et al., 1990; Shalaby, 1996; Kuley et al., 2011). Biogenic amines are toxic substances and are attributed with producing diseases in humans and animals, especially with respect to histamine and tyramine (Til et al., 1997; European Food Safety Authority

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[EFSA], 2011). In addition, some amines may be nitrosated or act as precursors for other compounds capable of forming nitrosamines, which are carcinogenic to various animals and a potential hazard for humans (Shalaby, 1996).

Cold-smoked fish products have received attention in terms of chemical and microbiological safety, from studies on detection and quantification of pathogenic microorganisms such as *Listeria monocytogenes* using technological strategies to ensure quality and safety (Vaz-Velho et al., 2005, 2006; Calo-Mata et al., 2008; Vermeulen et al., 2011; Todorov et al., 2012) to investigations on spoilage characterization examining the presence of biogenic amines (Jørgensen et al., 2000a; Joffraud et al., 2001; Chytiri et al., 2004; Dalgaard et al., 2008; Fadhlaoui-Zid et al., 2012). Identification of the spoilage microflora and biogenic amine production of single and cocultures growing in cold-smoked salmon was also studied by Jorgensen et al. (2000b), and these have been used as chemical indicators of seafood quality (Jørgensen et al., 2000a; Ozogul and Ozogul, 2006; Fadhlaoui-Zid et al., 2012).

Strains of Enterobacteriaceae and lactic acid bacteria (LAB) are the predominant microflora associated with cold-smoked fish and identified as active amine producers (Leroi et al., 2000; Jorgensen et al., 2000a; Silva et al., 2002). Jørgensen et al. (2000a) found *Photobacterium phosphoreum* to be primarily responsible for the production of biogenic amines in vacuum-packed cold-smoked salmon, where agmatine (160–220 mg/kg), cadaverine (260–470 mg /kg), histamine (100–220 mg/kg) and tyramine (50–130 mg/kg) were formed at 5°C.

In the Portuguese commercial market the majority of the vacuum-packed cold-smoked salmon originate from other European countries. The differences in origins of raw material and European producers make shelf lives quite different. In Portugal the production of cold-smoked fish is almost associated with cold-smoked salmon (*Salmo salar*) and salmon-trout (*Oncorhynchus mykiss*), with the latter raw material being produced in the north of Portugal. However, little information is available on microbial characterization of cold-smoked fish products available in the Portuguese market, their microbial variability, and their influence on biogenic amines production. This information is important due to their potential impacts on human health and food quality. The main objective of this study was to determine microbial characterization and quality of cold-smoked fish products available in the Portuguese market, and their potential to produce biogenic amines. Characterization of the microbiological profile during storage and at the end of shelf life, and studies on batch variability, levels of microbial hygiene indicators, and the related microbial quality were performed based upon European guidelines for commercial vacuum-packed cold-smoked salmon available on the market. The potential of bacterial isolates to produce biogenic amines was also examined.

MATERIALS AND METHODS

Smoked Fish Samples

Fish samples were obtained from Portuguese retail market (experiment I and experiment II) and for experiment III, directly from Portuguese producers at the end of production day. Cold-smoked salmon (*Salmo salar*) and cold-smoked salmon-trout (*Oncorhinchus mykssis*) were stored under refrigerated conditions (5°C). Samples analyzed were sliced fish fillets stored under vacuum packing. In each experiment, all samples were microbiologically analyzed every week, until the expiry date (experiment I) and during 4 wk of storage (experiments II and III).

Microbiological Analysis

From each pack, 30 g of cold-smoked fish was taken aseptically (10 g + 10 g + 10 g, from 3 different parts of the sample) and homogenized for 90 s in a stomacher (Seward 400). Ten grams of the mixture was aseptically taken and decimally diluted in sterile Maximum Recovery Diluent (CM 733; Oxoid) and homogenized for

20 s. Aerobic plate counts were performed on spread plates of Long and Hammer's medium (LH) (Van Spreekens, 1974) with additional 1% w/v NaCl, incubated at 15°C for 5–7 d. Counts of lactic acid bacteria (LAB) were obtained from pour plates of NAP medium, pH 6.7 (Davidson & Cronin, 1973), incubated anaerobically (Anaerocult A, Merck) at 21°C for 5 d. Enterobacteriaceae counts were obtained from pour plates of 5 ml tryptone soya agar (TSA), and after 2-h resuscitation of damaged cells at 20–25°C plates were overlaid with 12–15 ml of violet red bile glucose agar (VRBGA). Typical Enterobacteriaceae colonies were counted after 2 d of incubation at 30°C. To assess the selectivity of the different media, representative colonies were selected from plates and the following tests were performed: cell morphology, gram stain, and catalase and oxidase tests.

Bacterial Isolates

Representative colonies that grew on Long and Hammer's plates were selected and maintained on nutrient agar slants and stored at 5°C. Identification of the LAB group was based on the gram reaction, absence of catalase and cytochrome oxidase, and fermentative catabolism of glucose. For selected strains, API 20E (BioMérieux) and API 50CHL (BioMérieux) were used for identification of gram-negative strains and LAB, respectively.

Determination of Decarboxylase Capability

Thirty-two gram-negative and 22 strains that were included in the LAB group were selected to investigate the decarboxylating ability. The medium used to detect decarboxylating strains was prepared as described by Niven et al. (1981) and Silva et al. (2002). Strains were prepared for testing by subculturing in nutrient broth (NB) (Lab M) supplemented with 0.4% (w/v) each of the amino acids histidine and tyrosine, and incubated at 25°C for 48 h. A portion of each culture was spread on the decarboxylation agars, which were then incubated anaerobically (Anaerocult A, Merck) at 25 or 5°C for 48 h and 10 d, respectively. A purple halo was interpreted as positive for amine production. All strains were tested twice on separate occasions.

Examination of Proteolytic and Lipolytic Activity

Milk agar (10%) (Sigma-Aldrich) and tributyrin agar (Merck) were used to select bacteria that have proteolytic and lipolytic activity, respectively. The positive reaction was interpreted by a presence of a translucent halo surrounding the colonies.

RESULTS

Products and Storage Characteristics

The cold-smoked fish samples were in general exposed in the market at 5 ± 1°C in a commercial refrigerator open to consumers. The shelf life of the samples varied from 2 to 6 wk and, with the exception of one sample that was from Scotland, all others were from Norway (Table 1). Samples that originated directly from Portuguese smokehouses were stored at 4 ± 1°C. It was observed that one Portuguese smokehouse introduced a previous step of rapid freezing at –20°C after production, to improve the slicing process, and before chill commercialization. None of the samples showed any visual changes at the moment of collection.

Microbial Numbers and Characteristics

The microbiological characteristics of cold-smoked salmon samples from different producers and countries available on the Portuguese market are shown in Table 1. In general, microbial numbers increased during storage, with the exception of numbers of Enterobacteriaceae, which showed low initial numbers, with the exception of producer B (sample in the first week of storage) and producer M (sample at 2 wk of storage). At the end of shelf-life date

TABLE 1. Microbiological Characteristics of Cold-Smoked Salmon Samples Collected at Portuguese Retail Market

Producer	Country of producer	Raw fish origin	Time to expiry date (days)	Week of storage at chill conditions	pH	APC*	LAB*	Enterobacteriaceae*	Microbiological quality of cold-smoked salmon samples at expiry date**
Plant A	France	Norway	11	1	5.669	5.48	5.48	0.95	
				2	5.837	6.54	6.48	1.00	Acceptable
Plant B	Spain	Norway	31	1	5.856	5.48	5.56	3.54	
				2	6.065	4.00	3.00	1.00	
				3	6.094	6.48	5.54	0.00	
				4	6.320	4.85	4.00	0.00	Good
Plant C	Spain	Scotland	33	1	5.897	5.48	5.48	0.00	
				2	5.915	6.28	6.54	1.30	
				3	5.754	8.48	7.24	0.00	
				4	6.132	7.23	7.52	0.00	Unsatisfactory
Plant M	Portugal	Norway	10	1	—	—	—	—	
				2	5.827	4.30	3.48	0.00	Good
Plant M	Portugal	Norway	10	1	—	5.29	4.29	2.47	
				2	5.830	6.54	4.48	3.90	Acceptable
Plant F	Portugal	Norway	12	1	—	—	—	—	
				2	5.737	4.00	3.00	0.70	Good

*The numbers are average of duplicate counts in cfu/g of fish of APC (aerobic plate count), LAB (lactic acid bacteria), and Enterobacteriaceae.

**Based on microbiological quality guide for ready-to-eat foods, a guide to interpreting microbiological results (Food Standards Australia New Zealand, 2009) and on guidelines for assessing the microbiological safety of ready-to-eat foods placed on the market (Health Protection Agency, 2009).

and based on "Quality Guide for Ready-to-Eat Foods, A Guide to Interpreting Microbiological Results" (references for APC: good, $<10^6$; acceptable, $<10^7$ and $\geq 10^6$; unsatisfactory, $\geq 10^7$; references for Enterobacteriaceae group: good, $<10^2$; acceptable, $\geq 10^2$ and $<10^4$; unsatisfactory, $\geq 10^4$) (Food Standards Australia New Zealand, 2009), the results of microbial quality revealed one sample as "unsatisfactory," two samples "acceptable," and three samples classified as "good" (Table 1). A numerical increase of pH occurred in all samples during storage. In order to understand the variability of the microbial quality of cold-smoked fish products available on the Portuguese market (within the same producer and batch and between different producers), characterization of samples was performed. Data are shown in Table 2. Generically for the first 2 wk of storage, the coefficient of variation (CoV) of samples is high, mainly for the Enterobacteriaceae group and less for other groups of microorganisms (Table 2). The presented findings are an average of the group of samples in each week of sampling, for determination of the CoV, and consequently did not differentiate microbiological quality of samples within the same group. Although not shown in Table 2, some samples were beyond the limit of acceptance criteria for microbiological quality, even before the expiry date, based on the "Guidelines" (Food Standards Australia New Zealand, 2009; Health Protection Agency [HPA], 2009): Plant F, wk 2 (APC: 6.52 ± 1.31 CFU/g; Enterobacteriaceae: 2.11 ± 2.07 CFU/g), Plant F, wk 2 (APC: 7.48 ± 0.00 CFU/g; Enterobacteriaceae: 5.30 ± 0.37 CFU/g), and Plant Sp, wk 1 (Enterobacteriaceae: 4.68 ± 0.64 CFU/g).

The analysis of cold-smoked salmon collected directly at the end of production in Portuguese smokeries and cold-stored for 4 wk under lab-controlled time and temperature storage conditions is presented in Table 3. The CoV of these particular samples was lower compared to data in Table 2. In addition, the numbers of Enterobacteriaceae were considerably reduced in products from Portuguese plants S and M, compared to the other plants. Even though the CoV were in some cases higher than 20%, in general these values were essentially related to samples at the same storage period (first 2 wk).

Biogenic Amines Production by Bacteria

Bacterial strains were selected based on predominant colonies on agar plates from different storage times and from different producers. Tables 4 and 5 identified the amines producer bacteria, revealing positive reactions to tyramine and histamine based on appearance of a purple color around the colonies. Further identification to the genus and/or species level and its proteolytic and lipolytic activities are also shown. The results indicated a low percent of bacterial strains that displayed proteolytic activity (less than 5% and less than 13% for bacteria that were gram-positive and gram-negative, respectively). Higher percentages for lipolytic activity were observed (31% and 19% for bacteria that were gram-positive and gram-negative, respectively).

Overall, data indicated that the number of tyramine producers was higher than the number of histamine producers. At 25°C, gram-negative strains showed positive reactions for histamine and tyramine production, 40.6% (13/32) and 68.8% (22/32), respectively. At lower temperatures (5°C), these values decreased to 31.3% (10/32) and 37.5% (12/32), respectively. Bacteria responsible for this were identified as *Serratia liquefaciens*/*S. marcescens* and *Enterobacter* spp. For gram-positive strains, the findings at 25°C displayed percentages of 21% (4/19) and 47.4% (9/19) for histamine and tyramine production, respectively. At 5°C, only tyramine-producing strains were detected (16.7%; 3/18).

DISCUSSION

Commercial cold-smoked salmon and salmon-trout are the major smoked fish products available on the Portuguese market. In the last few years several studies have focused on characterization of microbiological patterns

TABLE 2. Microbiological Characteristics and Coefficient of Variation of Cold-Smoked Fish Samples Collected in the Portuguese Retail Market and Chill Stored During 4 wk in Laboratory Controlled Conditions

						APC*		LAB*		Enterobacteriaceae*	
Producer	Raw fish origin	Raw fish	Time to expiry date (days)	Number of samples (*n*)	Week storage	Average ± SD	Coefficient of variation (%)	Average ± SD	Coefficient of variation (%)	Average ± SD	Coefficient of variation (%)
Plant F	Norway	*Salmo salar*	18	$n = 6$	1	3.67 ± 1.23	33.6	2.64 ± 0.54	20.6	0.78 ± 0.66	85.3
				$n = 6$	2	6.52 ± 1.31	20.0	4.07 ± 0.56	13.6	2.11 ± 2.07	98.1
				$n = 7$	3	6.02 ± 0.22	3.70	4.75 ± 0.64	13.6	3.60 ± 1.06	29.4
				$n = 5$	4	7.43 ± 0.18	2.40	6.35 ± 1.34	21.1	1.74 ± 0.01	0.80
Plant F	Portugal	*Oncorhynchus mykiss*	19	$n = 4$	1	6.22 ± 0.35	5.60	5.25 ± 0.28	5.30	3.43 ± 0.11	3.20
				$n = 4$	2	7.48 ± 0.00	0.00	6.48 ± 0.00	0.00	5.30 ± 0.37	25.0
				$n = 4$	3	7.11 ± 1.29	18.2	5.62 ± 1.86	33.2	4.25 ± 0.33	0.00
				$n = 2$	4	7.91 ± 0.93	11.8	6.96 ± 0.05	0.70	1.74 ± 0.01	17.1
Plant S	Norway	*Salmo salar*	16	$n = 4$	1	4.47 ± 0.99	22.0	3.80 ± 1.18	31.1	1.64 ± 1.75	106.4
				$n = 2$	2	6.54 ± 1.33	20.3	4.48 ± 0.00	0.00	3.90 ± 0.82	21.0
				$n = 2$	3	6.61 ± 0.24	3.60	5.85 ± 0.22	3.80	4.00 ± 0.00	0.00
				$n = 0$	4	nd	nd	nd	nd	nd	nd
Plant M	Norway	*Salmo salar*	10	$n = 7$	1	5.60 ± 0.63	11.2	5.10 ± 0.36	7.10	2.23 ± 1.22	54.4
				$n = 2$	2	4.00 ± 0.00	0.00	3.00 ± 0.00	0.00	0.85 ± 0.21	25.0
				$n = 2$	3	4.67 ± 0.27	5.80	4.48 ± 0.00	0.00	1.00 ± 0.00	0.00
				$n = 2$	4	6.02 ± 0.65	10.8	5.39 ± 0.00	0.00	3.11 ± 0.53	17.1
				$n = 4$	1	5.83 ± 0.92	15.8	0.00 ± 0.00	0.00	4.68 ± 0.64	13.7
				$n = 4$	2	3.44 ± 0.37	10.8	1.00 ± 2.00	2.00	4.00 ± 0.00	0.00
Plant SP	Norway	*Salmo salar*	34	$n = 4$	3	2.96 ± 0.73	24.7	3.07 ± 0.16	5.20	2.06 ± 1.60	77.7
				$n = 4$	4	6.52 ± 0.08	1.20	3.00 ± 0.00	0.00	5.48 ± 0.00	0.00

Note. nd, Not determined.
*The numbers are average of samples counts in cfu/g of fish of APC (aerobic plate count), LAB (lactic acid bacteria) and Enterobacteriaceae.

TABLE 3. Microbiological Characteristics and Coefficient of Variation of Portuguese Cold-Smoked Salmon (*Salmo salar*) Samples Collected Directly From the Smokeries and Chill Stored During 4 wk at Laboratory Controlled Conditions

						APC*		LAB*		Enterobacteriaceae*	
Producer	Raw fish origin	Raw fish	Time to expiry date (days)	Number of samples (*n*)	Week storage	Average ± SD	Coefficient of variation (%)	Average ± SD	Coefficient of variation (%)	Average ± SD	Coefficient of variation (%)
Plant F	Norway	*Salmo salar*	24	$n = 3$	1	2.83 ± 0.35	12.5	2.56 ± 0.11	4.40	1.39 ± 0.66	31.5
				$n = 3$	2	5.36 ± 1.20	22.3	4.01 ± 1.00	25.0	2.44 ± 2.07	27.0
				$n = 3$	3	6.05 ± 1.20	19.9	5.27 ± 0.30	5.80	3.17 ± 1.06	14.1
				$n = 3$	4	7.36 ± 0.00	0.00	7.07 ± 0.00	0.00	5.20 ± 0.01	0.00
Plant S	Norway	*Salmo salar*	24	$n = 3$	1	2.48 ± 0.00	0.00	1.08 ± 1.52	14.4	0.00 ± 1.75	0.00
				$n = 3$	2	5.74 ± 0.18	3.10	5.00 ± 0.21	4.20	1.27 ± 0.82	30.1
				$n = 3$	3	7.07 ± 0.16	2.20	6.67 ± 0.27	4.00	1.00 ± 0.00	0.00
				$n = 3$	4	7.45 ± 0.07	0.00	7.30 ± 0.49	6.70	0.50 ± 0.00	141
Plant M	Norway	*Salmo salar*	32	$n = 3$	1	1.95 ± 0.49	25.4	0.00 ± 0.00	0.00	1.00 ± 0.00	0.00
				$n = 3$	2	3.20 ± 1.69	52.9	1.52 ± 0.74	48.4	1.00 ± 0.00	0.00
				$n = 3$	3	4.64 ± 0.34	7.30	3.05 ± 0.06	2.10	1.00 ± 0.00	0.00
				$n = 3$	4	6.46 ± 0.00	0.00	7.32 ± 0.00	0.00	1.00 ± 0.00	0.00

*The numbers are average of samples counts in cfu/g of fish of APC (aerobic plate count), LAB (lactic acid bacteria) and Enterobacteriaceae.

TABLE 4. Production of Biogenic Amines (Histamine and Tyrosine) and Proteolytic and Lipolytic Activities of Bacterial Gram (–) Strains Isolated From Cold-Smoked Salmon Commercial Samples

			Decarboxylation agar (Niven et al., 1989)						
			Histamine		Tyramine				
Strain designation	Source	Storage week	25°C (48 h)	5°C (10 d)	25°C (48 h)	5°C (10 d)	Milk agar proteolytic activity	Tributyrin agar lipolytic activity	Bacteria identification API 20 E
Gram (–)									
1	Plant B (salmon)	2	–	wg	–	wg	–	wg	—
2	Plant B (salmon)	2	–	wg	+	wg	–	–	—
3	Plant A (salmon)	3	–	wg	+	wg	wg	–	—
4	Plant A (salmon)	4	–	wg	+(*)	wg	wg	–	—
5	Plant B (salmon)	4	–	wg	+	wg	wg	–	—
7	Plant B (salmon)	4	+	+/–	–	wg	–	–	*Enterobacter* spp.
13	Plant B (salmon)	4	–	wg	+	wg	wg	–	—
14	Plant B (salmon)	4	+	+/–	+(*)	+	++	–	*Serratia liquefaciens/Serratia marcescens*
15	Plant B (salmon)	4	–	wg	–	wg	wg	wg	—
16	Plant B (salmon)	4	+	+/–	–	–	+	–	—
17	Plant B (salmon)	4	+	+/–	+	+/–	++	–	*Serratia liquefaciens/Serratia marcescens*
18	Plant B (salmon)	4	+	+/–	+	+/–	++	–	*Serratia liquefaciens/Serratia marcescens*
54	Plant M (salmon)	1	–	wg	+	+/–	+/–	–	—
55	Plant M (salmon)	1	+	wg	+	+/–	–	++	—
56	Plant M (salmon)	1	+	wg	+	+/–	–	–	—
58	Plant S (salmon)	1	–	wg	+	+/–	wg	–	—
59	Plant M (salmon)	1	–	wg	+	+/–	–	–	—
60	Plant M (salmon)	2	+	+/–	–	wg	–	–	*Enterobacter agglomerans*
61	Plant M (salmon)	2	–	wg	+	wg	wg	–	—
63	Plant F (salmon)	2	–(*)	wg	+(*)	wg	–	++	*Acinetobacter/Pseudomonas* spp.
66	Plant M (salmon)	3	–	wg	–	wg	–	++	—
67	Plant M (salmon)	3	+	wg	–	wg	–	–	—
68	Plant M (salmon)	3	+	+/–	–	wg	–	–	—
69	Plant F (salmon)	3	–	wg	+	wg	–	–	—
70	Plant F (salmon)	3	–	wg	+(*)	+/–	–	–	*Acinetobacter/Pseudomonas* spp.
71	Plant S (salmon)	3	–	wg	+	wg	–	–	*Acinetobacter/Pseudomonas* spp.
72	Plant S (salmon)	3	–	–	+(*)	wg	wg	++	*Pseudomonas fluorescens/putida*
73	Plant M (salmon)	1	–(*)	wg	+(*)	wg	–	–	*Acinetobacter/Pseudomonas* spp
74	Plant M (salmon)	1	+(*)	wg	+(*)	+	–	–	—
76	Plant F (salmon)	1	–	+/–	–	+/–	–	–	—
78	Plant M (salmon)	4	+	+	–	wg	–	–	*Enterobacter agglomerans*
79	Plant M (salmon)	4	+	+	+	+/–	++	–	*Serratia liquefaciens/Serratia marcescens*

Note. nd: Not determined; (+) and (–): positive and negative reaction, respectively, on decarboxylation agar (Niven et al., 1981), on milk agar and on trybutirin agar; wg: weak growth.
*Biogenic amines production confirmed and quantified by HPLC methodology (Silva et al., 2002).

TABLE 5. Production of Biogenic Amines (Histamine and Tyrosine) and Proteolytic and Lipolytic Activities of Bacterial Gram (+) Strains Isolated From Cold-Smoked Salmon Commercial Samples

			Decarboxylation agar (Niven et al., 1989)						
			Histamine		Tyramine				
Strain designation	Source	Storage week	25°C (48 h)	5°C (5 d)	25°C (48 h)	5°C (5 d)	Milk agar proteolytic activity	Trybutirin agar lipolytic activity	Bacteria identification API 50 CH
Gram (+)									
21	Plant A (salmon)	3	+	−	+	−	−	−	—
22	Plant A (salmon)	3	nd	nd	nd	nd	+	nd	—
28	Plant B (salmon)	4	+	−	+	−	−	nd	—
29	Plant B (salmon)	4	+	nd	−/+	nd	nd	+	—
31	Plant B (salmon)	4	−	−	+	−/+	–	–	—
32	Plant B (salmon)	4	+	nd	−/+		−	−	—
34	Plant F (salmon)	4	−	−	+(*)	−/+	–	–	*Carnobacterium divergens*
35	Plant B (salmon)	4	nd	nd	nd	nd	−	+	—
36	Plant S (salmon)	1	−	−	−	−	–	–	*Lactobacillus lactis* lactis
37	Plant S (salmon)	1	−	−	−	−	–	–	*Lactobacillus lactis* lactis
38	Plant S (salmon)	1	−	−	−	−	–	nd	—
39	Plant S (salmon)	2	−	−	−	−	–	nd	—
41	Plant M (salmon)	3	−	−	+(*)	−	–	+	*Lactobacillus lactis* lactis
42	Plant F (salmon)	3	+	−	+	−	–	nd	—
43	Plant S (salmon)	3	nd	nd	nd	nd	–	+	—
44	Plant S (salmon)	3	−	−	+	−	–	nd	—
47	Plant M (salmon)	4	−	−	−	−	–	−	—
48	Plant M (salmon)	4	−	−	−	−	–	–	—
49	Plant S (salmon)	4	−	−	+	−	–	+/−	—
C	Plant M (salmon)	1	−	−	+	−	–	–	—
D	Plant S (salmon)	1	−	−	−	−	–	–	—
101	Plant S (salmon)	4	+/−	−	−	−	–	–	—

Note. nd: Not determined; (+) and (–): positive and negative reaction, respectively, on decarboxylation agar (Niven et al., 1981), on milk agar and on trybutirin agar; wg: weak growth.
*Biogenic amines production confirmed and quantified by HPLC methodology (Silva et al., 2002).

and spoilage processes, and on control of pathogenic bacteria, such as *L. monocytogenes*, in cold-smoked fish. EFSA (2011) indicated that the "safety of consumers" was dependent not only on the presence of this pathogen but also on levels of the biogenic amines tyramine and histamine produced by decarboxylation of amino acid precursors. Thus, evaluation of the presence of these biogenic amines in cold-smoked fish products and identification of the biogenic amino producer's bacteria are of concern for public health, consumers, and producers. In fact, it is known that microbial activity is responsible for spoilage and quality deterioration of these products, and as the presence of biogenic amines is dependent on the composition of the microflora, it is important to ascertain the sources of this microflora, including poor hygiene practices, preservation and technological procedures, and storage conditions (Food and Drug Administration [FDA], 2004; EFSA, 2011; Fadhlaoui-Zid et al., 2012). In this study, the CoV of the samples indicated that the microbial quality was not homogeneous within a batch, and at the expiry date samples showed different levels of contamination. Several technological factors, such as salting and smoking procedures, and packaging conditions, in combination with storage temperatures and hygiene procedures, exert a direct influence on microbial characteristics and microbial growth (Leroi et al., 2000; Cid et al., 2008; EFSA, 2011; Zhai et al., 2012). Data indicated that the storage and temperature conditions influenced microbial growth, a fact easily observed when comparing samples stored in controlled temperature conditions in the lab that present low CoV compared to the ones stored in retail market conditions. In this study, LAB and Enterobacteriaceae were the predominant groups present in commercial vacuum-packed cold-smoked fish, and this is in agreement with other previous studies (Leisner et al., 1994; Hansen, 1995; Jorgensen et al., 2000b). However, some differences in the behavior of LAB and Enterobacteriaceae in products were observed among the different producers. The results demonstrated that the CoV of samples decreased with storage time (Tables 2 and 3). Further, the CoV of samples was higher for Enterobacteriaceae and aerobic plate count, especially in the first week of storage, with values also declining correlated to further storage time. Data suggest that the determinant factors influencing microbial characteristics, such as raw material quality and technological process (salting and cold-smoking), in combination with good manufacturing practices, exert major influences on the quality attributes of the final product. However, storage temperature fluctuations, which may occur at distribution and retail points, also exert a considerable influence. These results are in agreement with Dondero et al. (2004), who demonstrated that the quality and shelf life of cold-smoked salmon was a function of storage temperature control. The findings also showed that a significant number of bacteria present on commercial Portuguese cold-smoked salmon produce tyramine and histamine, dependent on the producer and week of storage. The presence of high levels of LAB and Enterobacteriaceae in products may be associated with the presence of biogenic amine-producing bacteria, since some species of these groups are indicated as biogenic amines producers (Jorgensen et al., 2000b; Cid et al., 2008; Curiel et al., 2011; Bunka et al., 2013). Most of these Enterobacteriaceae belong to the genera *Serratia* and *Enterobacter*, the most frequently isolated from cold-smoked salmon (Hansen and Huss, 1995; Jorgensen et al., 2000a; Silva et al., 2002). The higher LAB counts generally present in these products, in conjunction with Enterobacteriaceae (or other spoilage microorganisms e.g. *Photobacterium phosphoreum*), seem to be a general occurrence. Hansen (1995) indicated that producers need to pay special attention to the inclusion of a contaminating flora via fair hygiene conditions and poor manufacturing practices that might contribute to heavy spoilage and biogenic amines production. In addition, *Lactobacillus curvatus* was identified as a specific spoilage organism in cold-smoked salmon, probably with a spoilage domain different from *P. phosphoreum* (Jorgensen et al., 2000a; Dalgaard et al., 2008). In addition to tyramine and histamine

production, some strains displayed proteolytic activity that may contribute to the production of biogenic amines, due to the availability of proteins and amino acids in fish muscle. In our study the results were positive for the presence of the two bacterial strains, *Serratia liquefaciens*/*Serratia marcescens*. Data demonstrated that some samples before the expiry date were microbiologically unsatisfactory, presenting high microbial numbers for aerobic plate counts or presenting high numbers of the Enterobacteriaceae group, indicating poor microbiological quality. Further, a high percentage of tyramine-producing bacteria and some histamine producers suggested that the microbial characteristics of samples were not homogeneous within a producer (and batch), which implies that improvements to standardize methods and procedures in cold-smoked fish production need be considered, as well as procedures monitoring the hygiene and storage conditions at distribution and retail level.

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CHAPTER 4.

Challenge studies on cold-smoked fish production and their relation to spoilage and safety

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Inactivation by ozone of *Listeria innocua* on salmon-trout during cold-smoke processing

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Abstract

This study was conducted to evaluate the efficacy of gaseous ozone exposure on *Listeria innocua* 2030c growth during cold-smoke processing of *Oncorhynchus mykiss* (salmon-trout). Three sets of experiments were performed: inoculation with *L. innocua* 2030c followed by a 20 min ozone exposure were applied to (a) fresh salmon-trout after filleting, (b) to fresh whole fish, and (c) to fresh whole fish after removal of the fish slime. In sets (a) and (b) fillets were subsequently cold-smoked but not in set (c). The ozone concentration inside the exposure chamber after 20 min reached 0.1×10^{-3} g/l.

Counts of *L. innocua* 2030c were performed after treatment for sets (a), (b) and (c), after smoking and weekly during 21 days in vacuum packs at 5 °C, for sets (a) and (b). Sampling for total Aerobic Plate Counts (APC) of non-inoculated samples was also performed in set (a). The percentage of salt in the water phase, peroxide values and the effect of treatment on sensory properties of cold-smoked salmon-trout fillets were also determined in the first and second set. In the first set, a decrease of less than 1 $\log_{10}$ in *L. innocua* numbers occurred on ozone treated samples in all sampling occasions. APC was slightly lower on fresh fillets after treatment and on smoked fillets at 3 weeks of storage at 5 °C (less than 1 $\log_{10}$ CFU/g). In the second set, a reduction greater than 1 $\log_{10}$ *L. innocua*/g occurred on smoked fillets at the end of the storage period. In the third set, the slime removal resulted in a 1 $\log_{10}$ *L. innocua*/g reduction on fresh treated samples.

Ozone treatment had no significant ($p > 0.05$) effect on *L. innocua* counts on samples compared to those on untreated fish. In both sets, no significant differences among ozone treated/untreated samples were noticeable by sensory evaluation ($p > 0.05$).

Keywords: *Listeria* spp.; Ozone; Cold-smoked fish

1. Introduction

Listeria monocytogenes is an ubiquitous psychrotrophic bacterium responsible for foodborne infections worldwide. Human listeriosis is predominantly a foodborne disease caused by *L. monocytogenes*, and although rare, has a high mortality. The disease most often affects unborn or newly delivered infants, pregnant women, and the immunocompromised (Farber & Peterkin, 1991). The pathogen has been consistently isolated from production lines of fresh to cold-smoked fish (e.g., Ben Embarek, 1994; Dillon, Patel, & Ratnam, 1992; Eklund et al., 1995; Farber, 1991; Jemmi & Keusch, 1994; Johansson, Rantala, Palmu, & Honkanen-Buzalski, 1999; Jørgensen, 2000; Jørgensen & Huss, 1998; Rørvik, Caugant, & Yndestad, 1995; Vaz-Velho, Duarte, &

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Gibbs, 2000; Vaz-Velho, Duarte, & Gibbs, 1998). A number of small outbreaks associated with smoked fish and shellfish have also been reported (Brett, Short, & McLauchlin, 1998; Ericsson et al., 1997; Hall, Pelerin, Soltanpoor, & Gilbert, 1995; Miettinen et al., 1999; Misrachi, Watson, & Coleman, 1991; Mitchell, 1991). The mild temperatures (<30 °C) and low salt concentrations [<5% (water phase salt)] used in the cold-smoked processing of fish are not sufficient to inactivate *L. monocytogenes*. Growth of the organism may occur in the product during storage and cold smoked fish does not undergo cooking before consumption (final report of Spoilage and safety of cold-smoked fish, 2000).

The use of ozone as a surface disinfectant of meats (Greer & Jones, 1989; Sheldon & Brown, 1986) and for the preservation of shrimps (Chen, Huang, Moody, & Jiang, 1992) has been reported. The extension of the shelf-life of perishable foods using ozone to reduce microbial activity has also been reported by Rice, Farquhar, and Bollyky (1982). As reported by Nelson (1982), the fresh quality of ozone-iced pacific salmon could be maintained for up to 6 days. The applications of ozone to fish preservation have been studied by several authors (e.g., Ravesi, Licciardello, & Racicot, 1987; Rice & Graham, 2001; Rice et al., 1982; Silva, Gibbs, & Kirby, 1998). Ozone concentrations as low as 0.01 ppm are toxic to bacteria and Gram-positive bacteria are more sensitive to ozone than Gram-negative bacteria (Mielcke & Ried, 2004).

In the United States ozone has received in 1997 GRAS (generally recognised as safe) classification, and in 2001 the FDA officially approved media containing ozone for use in the food industry, also for direct contact with food products, including fish, meat and poultry (Mielcke & Ried, 2004).

Until now, no information was available regarding the effect of ozone on *Listeria* spp. associated with cold-smoked fish processing. In this study, *L. innocua* 2030c, a tetracycline resistant strain, was used to replace the pathogen *Listeria monocytogenes* due to processing plant constraints and because all the *Listeria* strains isolated from Portuguese cold-smoked fish products were, to date, tetracycline sensitive (Vaz-Velho, Duarte, McLauchlin, & Gibbs, 2001). The behaviour of this strain, already studied, is similar to the major types of Portuguese *L. monocytogenes* strains (serotypes 4b and 1/2c) with respect to its growth/survival patterns under exposure to gaseous ozone. The best time/concentration combination of gaseous ozone for reducing *L. innocua* 2030c and *L. monocytogenes* 4b and 1/2c pure culture numbers was 20 min of exposure to ozone concentration of 0.1×10^{-3} g/l resulting in a 3–3.5$\log_{10}$ *Listeria* spp./ml reduction (Vaz-Velho, Fonseca, Silva, & Gibbs, 2001).

This remarkable effect of ozone on pure cultures of *Listeria* spp. created great expectations for challenge studies with cold-smoked fish processing. Therefore, the purpose of this study was to ascertain if the application of gaseous ozone to raw fish has any effect on *Listeria* numbers during cold-smoked processing and further chilled storage for 3 weeks at 5 °C in vacuum packs. Such treatment could be a potential measure for reducing *L. monocytogenes* numbers on products intended to be maintained "traditional" but safe.

2. Materials and methods

Fresh, premium quality salmon-trout (*Oncorhynchus mykiss*) with an average weight of 1.5 kg, farmed and slaughtered in the north of Portugal were placed in polystyrene boxes covered with ice and transported to the laboratory and stored overnight at 5 °C. The next day the fish were eviscerated, beheaded and filleted. Three sets of experiments were performed.

For the first set of experiments (a), inoculation and treatment were applied to fish after filleting and fillets were then cold-smoked. In the second experiment (b), inoculation and treatment were applied to the whole fish before filleting to avoid the potential interference of muscle exposure and, after filleting, fish was subsequently smoked. Another experiment (c), inoculating and treating the whole fish with or without the presence of slime, was performed. Slime was removed from the fish surface by washing the fish with water for 15 min before inoculation and treatment, but the fish fillets were not smoked.

2.1. Ozone generation and measurements

Ozone was produced by a domestic ozone generator model PR1 (TRIOZON, Spain) using atmospheric air as the source of oxygen. The ozonated air produced at a constant flow rate by the apparatus (0.76 l/min) was passed via a silicone tube to a pump, and then to a plastic chamber of 26 dm^3 volume. The ozone in the air flow produced by the apparatus (0.32 mg/l) was measured experimentally by the iodometric method as described Silva et al. (1998). In addition, the ozone concentration in the air flow inside the box was calculated as described by Silva et al. (1998). The ozone concentration inside the chamber after 20 min reached 0.1×10^{-3} g/l.

2.2. Ozone treatments

An overnight 20 ml culture (30 °C, 18 h) of *L. innocua* 2030c a tetracycline resistant strain from Public Health Laboratory Services (PHLS, London/UK) private collection, yielding 10^{8-9} CFU/ml in Tryptone Soy Yeast Extract agar (Tryptone Soy Broth + 6 g/l yeast extract + 12 g/l agar (TSB-YE, Lab M, Bury, Lancashire/UK)) was centrifuged (Centromix P Selecta, Spain;

2400 g, 10 min) and the cells resuspended in 20 ml of sterile 1% (w/v) NaCl (Merck, Darmstadt/Germany). This suspension was further diluted in 1 l of sterilised water with 1% of NaCl (w/v). Fish fillets (set a) or whole fish (sets b and c) were introduced into this bath for 30 s. A cell concentration of about 10^6, 10^4 and 10^6 CFU/g was obtained in the raw fish of set (a), set (b) and set (c), respectively.

In the first set of experiments (a) the trials were as follows:

Trial 1. Two inoculated fillets subjected to ozone exposure for 20 min.
Trial 2. Two inoculated fillets not subjected to ozone exposure.
Trial 3. Two fillets not inoculated but exposed to ozone for 20 min.
Trial 4. Two remaining fillets were not inoculated nor treated with ozone.

Fillets were left with the belly skin and the lug bone and were further dry-salted and cold-smoked (Vaz-Velho, Duarte, & Gibbs, 2001). After smoking the fillets were sliced and portions of slices were vacuum packed and stored at 5 °C. This experiment was performed three times (three smoking batches).

For the second experiment (b), where inoculation and treatment were applied to the whole fish, the procedure was as follows: two fish were inoculated, one fish being subjected to a 20 min ozone exposure and the other fish not treated; two fish were not inoculated, one fish being subjected to ozone treatment and the other fish not. Fish were filleted before salting and smoking. Number of trials and procedures were as in set (a) but just one smoking batch was performed.

A third experiment (c) was performed by inoculating and treating the whole fish before and after removal of the slime to evaluate whether slime on the raw fish might interfere with ozone activity. The procedure was as follows: two fish inoculated before removal of the slime, one being subjected to a 20 min ozone exposure and the other fish not; two fish inoculated after removal of the slime, one being subjected to a 20 min ozone exposure and the other fish not. Fish were filleted but not further smoked.

2.3. Sampling and enumeration

25 grams of fish fillets (fresh and smoked material) were homogenised with 225 ml of sterile Maximum Recovery Diluent (MRD, Lab M, Bury, Lancashire/UK) in a Stomacher (Seward 400) for 2 min. One milliliter of this suspension was placed in tubes containing 9 ml of MRD. Serial decimal dilutions to 10^{-4} were made in MRD.

Sampling was done after inoculation and/or ozone treatment, after smoking, and weekly during the 3 weeks of storage in vacuum packs at 5 °C. Two samples per treatment were analysed on each occasion.

Enumeration of *L. innocua* on fish fillets was by spread plating onto PALCAM agar (Merck, Darmstadt, Germany) with 8 μg/ml tetracycline–HCl (Sigma, Steinheim/Germany) incubated at 30 °C for 48 h before counting the typical colonies (black colonies with a dark halo). Total Aerobic Plate Counts (APC) were also determined in non-inoculated samples using Long and Hammer's (1941) plating medium (modified by Van Spreekens, 1974) incubated at 25 °C for 5 days.

2.4. Cold-smoking processes

For the first set of experiments (a), three batches of smoked fish were produced. Differences between the processes were for practical reasons in obtaining a reasonable time of salting/smoking and saving time in the experiment and still producing an acceptable commercial smoked product.

For both sets of experiments (a, b) dry-salting was done for 6 h at 16 °C, with a sugar:salt mix of 1:6 by weight. The weight of salt + sugar corresponded to one third of the weight of the fillet. After salting, the fillets were washed/rubbed with running water to remove the surplus of salt, and were hung to drain overnight at 5 °C. Smoking was done for 8, 6 and 5 h, respectively for the first, second and third batches of the first set (a) and for 5 h for the second experiment (b). A vertical smoker (AGK, Type 135/12, Wallersdorf, Germany) was used but a more sensitive thermostat and timer (lae® electronic, MTR12, Oderzo, Italy) replaced those supplied with the smoker. Smoke was produced by smouldering beech wood chips (Räucher Gold®, type Kl 2/16, Rettenmaier & Söhne, Rosenberg, Germany). Time/temperature profiles of the processes were recorded on a portable microprocessor (Hanna instruments, HI 92804c Bedfordshire, UK). Although smoke temperature occasionally exceeded 30 °C, temperatures of the fillets were always below 28 °C. Relative humidity, recorded by an hygrometer (Rotronic AM3, Knoxville, USA) decreased from 80% to 70% during the first three hours, decreasing further to 52% at the end of the smoking process.

The smoked fillets were cooled overnight at 5 °C. The following day, lug and pin bones and belly flaps were removed and the fillets were then sliced by hand. Portions of slices (skin off), of about 100 g each, were vacuum packed (Multivac Sepp Haggenmüller KG, A300/41/42, Germany; 1 mbar/10 s) and stored at 5 °C for weekly analysis. The permeability of the packs to O_2, CO_2 and N_2 were respectively 4, 13 and 4 mol/m^2 d bar.

2.5. Chemical analysis

Sodium chloride and moisture contents and peroxide values of the finished products were evaluated following respectively, the Portuguese NP 2929 (Fish and fish products—determination of chlorides content, 1988), NP 2282 (Fish and fishing products—determination of the water content (Reference Method), 1991) and NP 3142 (Fish and fish products—determination of peroxide value, 1990) protocols for fish products. These analyses were performed after 3 weeks of storage in vacuum packs at 5 °C.

2.6. Sensory evaluation

After 3 weeks storage at 5 °C, sensory evaluation of non-inoculated ozone-treated and untreated samples was carried out with 10 semi-trained panelists. Judges were asked to classify the samples for overall acceptability regarding the flavour and aroma, choosing one of these five hypotheses: I like very much; I like; I don't like nor dislike; I dislike, I dislike very much, scored respectively with 5, 4, 3, 2 and 1 points. Samples scored with totals of $\geqslant 30$ points were considered acceptable. Judges were requested to justify their classification specifying why they liked or disliked. The protocol used was that adopted by the EC FAIR Project CT/95 1207 "Spoilage and safety of cold-smoked fish".

2.7. Statistical analysis

The effect of ozone treatment on sensory properties of the smoked fillets, within the different batches of sets (a) and (b) at the end of the third week of storage, was evaluated by analysis of variance. The ANOVA test was applied for quantitative analysis of the taste panel results. The effect of ozone treatments on *L. innocua* 2030c and APC numbers within the different batches and for all sampling times was evaluated by analysis of variance (ANOVA). It was accepted there was a significant difference between ozone treated/untreated samples if $P < 0.05$.

3. Results and discussion

As previously mentioned, differences in smoking times of the three batches from the first set (a) were for practical reasons only, yet still producing an acceptable commercial-like smoked product. However, as no significant differences in *L. innocua* numbers and APCs were found among the three batches ($P > 0.05$) of set (a), the average was considered.

The effect of prior ozone exposure on growth of *L. innocua* 2030c and APC during the cold-smoke processing and storage of set (a) is shown in Figs. 1 and 2.

In the first experiment, and in the first batch, the % of salt in the water phase (w/w) was too high (average = 7%) and decreased to 5.7% and 5.8% respectively in the second and third batches. In the second experiment the average % of salt in the water phase was 5.8% (w/w). According to Huss, Embarek, and Jeppesen (1995), a commercially acceptable level of salt should be below 5% in the water phase and for safety reasons this percentage must be at least 3%. Although the amount of salt in the water phase exceeded the recommended 5%, as sugar was added to the salt in the proportion 1:6 sugar:salt, the saltiness was not noticeable. All the products had levels of salt in the water phase $\geqslant 3.5\%$ (w/w) (Tables 1 and 2) but *L. innocua* 2030c is known to grow well at those levels of salt (Vaz-Velho, Durate & Gibbs, 2001).

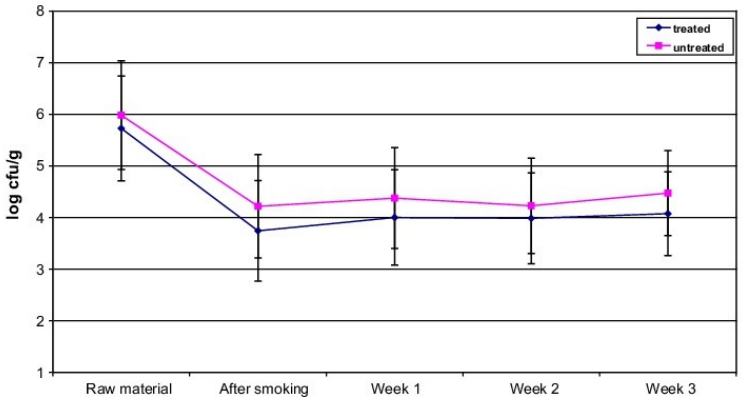

Fig. 1. The effect of gaseous ozone on *Listeria innocua* 2030c numbers during cold-smoke processing of salmon-trout (set a—ozone applied to fresh fish after filleting).

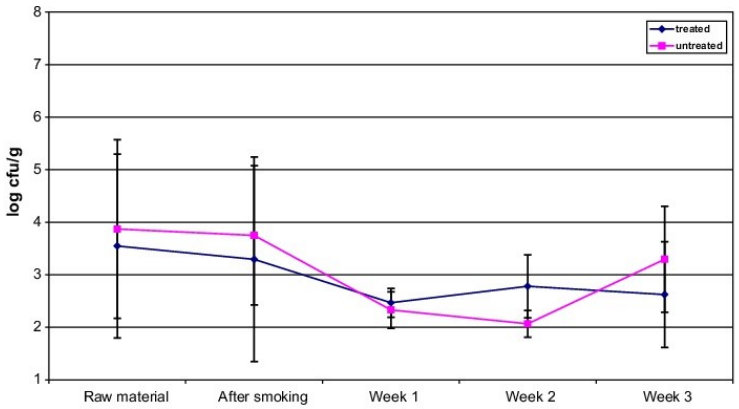

Fig. 2. Effect of gaseous ozone on APC during cold-smoking processing of salmon-trout (set b—ozone applied to fresh fish after filleting).

Table 1
First set (a)—sensory scores, peroxide values and % salt in the water phase of the three batches

Samples	Batch 1			Batch 2			Batch 3		
	Sensory scores	Peroxide value (meq/kg of fat)	%NaCl (w/w)	Sensory scores	Peroxide value (meq/kg of fat)	%NaCl (w/w)	Sensory scores	Peroxide value (meq/kg of fat)	%NaCl (w/w)
Inoc./treated	nd	2	7.1	nd	5	5.7	nd	Not detected	4.6
Inoc./treated	nd	3	8.3	nd	15	5.7	nd	3	5.4
Inoc./untreated	nd	Not detected	7.1	nd	7	5.5	nd	4	6.1
Inoc./untreated	nd	Not detected	6.1	nd	Not detected	6.2	nd	2	8.3
Treated	35	Not detected	4.7	43	Not detected	5.1	36	Not detected	5.7
Treated	nd	Not detected	8.7	nd	6	6.7	nd	Not detected	5.0
Untreated	39	Not detected	8.0	40	3	5.1	41	Not detected	4.4
Untreated	nd	Not detected	6.0	nd	Not detected	5.2	nd	3	6.7

nd: not determined.

Table 2
Second set (b)—sensory scores, peroxide values and % salt in the water phase

Sample	Panel score	Peroxide value meq/kg of fat	%NaCl (w/w)
Inoc./treated	nd	2.6	7.9
Inoc./treated	nd	10	8.8
Inoc./untreated	nd	3.6	6.7
Inoc./untreated	nd	6.1	3.8
Treated	nd	5.2	5.4
Treated	36	2.6	3.5
Untreated	38	2.3	4.3
Untreated	nd	10.7	6.4

nd: not determined.

Jørgensen (2000), found values of water phase salt in commercial Danish cold-smoked salmon ranging from 3% to 12.5%. Portuguese commercial cold-smoked swordfish always presented levels of salt (water phase salt) ≈8% (w/w) and *L. monocytogenes* has been consistently isolated from this kind of products (Vaz-Velho, Durate, McLauchlin et al., 2001).

In the first set of experiments (a) the best sensory scores were obtained by the untreated, treated and untreated samples respectively in the first, second and third batches (Table 1). In the second set (b) the untreated sample was scored best (Table 2). However, in both batches statistical analysis did not differentiate the products ($P > 0.05$).

No relationship between peroxide values and ozone treatment or moisture content was found in the samples tested, and the concentration of ozone used in this study did not significantly change the sensory characteristics of the smoked products.

As shown in Fig. 1, exposure to gaseous ozone at the levels used did not significantly reduce the numbers of *L. innocua* inoculated on salmon-trout fillet surfaces (a reduction of less than 1 $\log_{10}$ CFU/g in all sampling occasions). With respect to APC, although at the end of the storage period treated samples showed slightly

lower numbers (Fig. 2), no significant effect on APC was found—statistical analysis did not differentiate the treated/untreated products ($p > 0.05$).

The required ozone concentration and, of course, the disinfection rates depend in every case on the type of microorganism, on the degree of biological contamination, on the temperature, pH, turbidity and on the presence of ozone-oxidisable substances (Mielcke & Ried, 2004).

Restaino, Erampton, and Hemphill (1995), reported on the efficacy of ozone to reduce the numbers of *L. monocytogenes* and other pathogenic microorganisms. These authors showed that *L. monocytogenes* was more sensitive to ozonated water than the other Gram-positive and Gram-negative bacteria studied, either in the presence or absence of organic material.

Silva et al. (1998) reported gaseous ozone to be effective in reducing numbers of scad spoilage flora. These authors compared two ozone treatments on whole-eviscerated fresh fish: (i) the fish subjected just to a single ozone treatment before ice storage and (ii) subjected to a daily ozone treatment during iced storage. This last option, performed in the laboratory, was more effective in reducing the spoilage flora than the former. On the other hand, continuous ozonation of fish in boat holds (ozone rate maintained constant), was shown to be more effective than in the reported laboratory experiments, by increasing the lag phase and reducing the numbers of all tested microorganisms (Silva et al., 1998).

The interference of organic matter (exposure of fish muscle on fillets) was considered as the main potential reason for the results obtained in this first set of experiments. It has been reported that sensitivity of microorganisms to ozone is affected by several factors including the presence of organic matter (Glaze, 1986; Hoigné & Bader, 1975). A high and persistent level of organic substances will have a negative impact on ozone disinfection rate (Mielcke & Ried, 2004).

The applicability of ozone treatments in the cold-smoked fish industry, a major objective of this work, made it necessary to choose the best practicable time to apply the treatment to the fish. It was assumed for a smoking plant, the best moment would be after filleting and washing (less contamination by further handling) and just before salting.

In the literature reviewed, ozone treatments were applied to the whole fish rather than to fillets, hence the decision to treat whole salmon trout (set b). However, if this treatment shows greater potential for reducing *Listeria* numbers, the raw fish suppliers should apply the treatment before the fish arrive at the smoking plants, which may not be well-accepted by the fish farmers unless reliable raw material guarantees become mandatory.

As in set (a) ozone treatment did not significantly reduce *L. innocua* 2030c numbers at any stages of the processing chain and during storage, it was assumed that organic matter might have been the reason by interfering with ozone activity. The effect of ozone exposure on subsequent growth of *Listeria innocua* 2030c in the second experiment (b) is shown in Fig. 3. In this second experiment (b), fish was treated before filleting therefore the potential interference of the organic matter due to the exposure of the muscle was reduced; ≈1 log reduction in *L. innocua* numbers was found in the raw fish after treatment and at the end of the storage period this reduction was greater than 1 log (Fig. 3). No differences between treated/untreated samples were noticed after smoking until week 2 when treated fish showed higher numbers than untreated fish (≈1 log cycle) and, at week

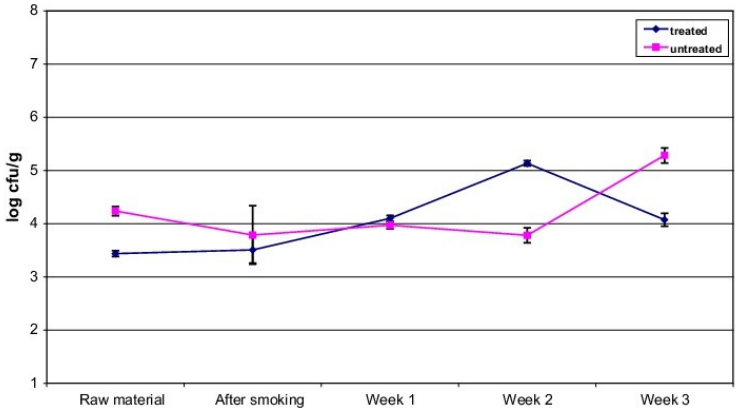

Fig. 3. The effect of gaseous ozone on *Listeria innocua* 2030c numbers during cold-smoke processing of salmon-trout (set b—ozone applied to fresh whole fish).

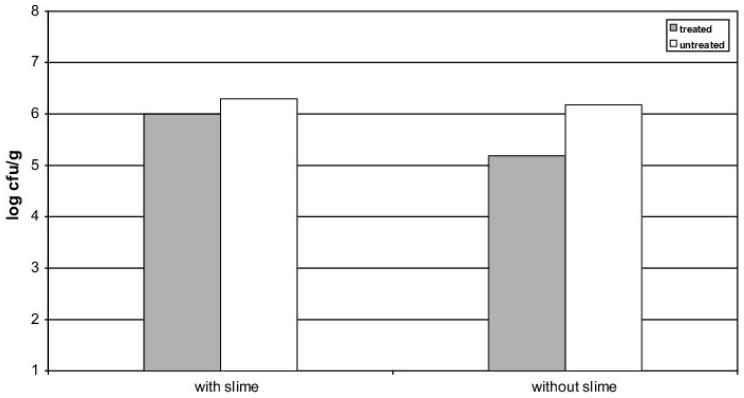

Fig. 4. The effect of gaseous ozone on *Listeria innocua* 2030c numbers in fresh whole salmon-trout with and without slime (set c).

3 the opposite happened. No clear explanation was found for these contradictory counts after 2 and 3 weeks of storage (standard deviations were very low). Statistical analysis did not differentiate the treated/untreated samples ($P > 0.05$).

Since it was possible that the slime layer present on salmon-trout skin but not in salt water fishes such as scad (Silva et al., 1998), might have reduced the efficacy of ozone treatment, a third experiment (c) with fresh whole salmon-trout was then performed. Inoculation with *L. innocua* and ozone treatments were applied to the fresh whole fish before and after removal of the slime layer, but the fillets were not then smoked. The results are presented in Fig. 4. When slime was removed before the inoculation and treatment ≈1 log reduction of *L. innocua* 2030c numbers on the raw fish was observed compared to the untreated samples. However, statistical analysis did not differentiate samples treated before removal of the slime from those treated after removal of the slime ($P > 0.05$).

Contrary to the results of this study, Rice and Graham (2001) reported ozone as a very effective microbicidal agent, reducing bacterial numbers in various types of food including fruits, vegetables, poultry and fish.

Previous studies using bacteria representative of normal fish spoilage flora (Silva et al., 1998) and *Listeria* spp. pure cultures (Vaz-Velho, Fonseca et al., 2001) exposed to various ozonation times, indicated that the survival rate of the tested bacteria was not linearly related to ozonation time, the higher death rate occurring during the first 15 and 20 min of exposure with a level of ozone inside the desiccators of about 0.25×10^{-3} g/l and 0.1×10^{-3} g/l respectively for the first and last studies. Of course, those experiments were performed on pure cultures of *Listeria* spp. or for reducing the total viable flora of smaller fish. For fish of greater dimensions such as salmon-trout, the length of exposure to ozone used in this study might have been too short. Additionally, care must be taken when using ozone applications to prevent the oxidation reactions that can occur making the product unacceptable. Since in the present study no relation between peroxide values and ozone treatment or moisture content was found in the samples analysed, higher concentrations of ozone or longer exposure, could be tested in future experiments.

The maintenance of a reliable and healthy food supply is the greatest challenge for food industry. Nowadays, it is possible to produce ozone in an economical and very reliable way—0.90 to 1.60 €/kg of ozone (Mielcke & Ried, 2004). The utilisation of ozone for direct contact with food products has been approved by FDA, in 2001, therefore it is likely that individual and special ozone application practices have been developed or will be processed quite soon.

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4.2. Does a previous freezing step at -20ºC before product commercialisation at chill conditions (5ºC) influence the microbial ecology of vacuum-packed cold-smoked salmon-trout (*Oncorhynchus mykiss*)?

Abstract

Evaluation an additional frozen step (-20ºC) after smoking as a potential factor to modify the microbial ecology of vacuum-packed cold-smoked salmon-trout (*Oncorhynchus mykiss*) that were previously salted by different procedures and smoked was performed in a pilot-scale trial. Differential Scanning Calorimetry (DSC) was used to study the effects of cold-smoking processing on stability of myofibrillar proteins. Microbiological and pH status during chill storage at 5 ºC were analysed weekly. Results demonstrated that microbiological numbers in end product subjected to a prior freezing process were in general higher for APC, LAB, *Enterobacteriaceae* and H_2S producing bacteria. This difference was evident in the first week of chill storage and maintained during storage for some subgroups of samples as a function of the salting process applied. Changes on myofibrillar proteins were observed after salting/cold-smoking process. The stability of myofibrillar proteins were affected by salt treatment and the additional freezing step can result in decrease in product quality. Samples that were previously frozen achieved a significant increase in microbiological numbers, such as H_2S-producing bacteria, indicating some caution on the application of this process after salting/smoking before market storage chill conditions <5ºC. This does not preclude the possibility of including the freezing step in the commercial process, which should be studied by checking their relevance, by controlling conditions (time and temperature) on production and/or on commercial market facilities.

4.2.1 Introduction

Food safety and quality is a growing preoccupation of health authorities and major food companies, including those who are operating in traditional and artisanal facilities, to guarantee the availability of safe products to consumers. Temperature is one of the most important factors related to the control of microbial growth and selection of different components of the microbiota of cold-smoked fish products (Dondero *et al*., 2004; Lyhs *et al*., 1998a, 1998b; Palludan-Muller *et al*., 1998; Silva and Gibbs, 2015). Psychrotrophic pathogens and spoilage bacteria are frequently found in raw materials and processing plants, where these microorganisms can survive and proliferate at refrigeration temperatures (Messi *et al*., 2003; Moreto *et al*., 2016). Studies indicated that a decrease of the storage temperature and an increase of salt concentration extended the storage life of cold-smoked salmon (Civera *et al.*, 1995; Hansen, 1995¸ Leroi and Joffraud, 2000; Lovdal, 2015; Gallart-Jornet *et al.*, 2007a, 2007b). At refrigeration temperatures (5ºC), the "lightly preserved" products, as classified cold-smoked fish, included salt content <6% NaCl (w/w) in the water phase (SWP) and low acid content (pH>5.0), resulting in a limited shelf-life (Huss, 1994; Gram *et al*., 2001b). The type of packaging and the SWP content imposes the interval of chill storage temperature of cold-smoked fish (FDA, 2015). The salt concentration affects the stability and denaturation of proteins and thereby the physicochemical factors such as water holding capacity (WHC) (Thorarinsdottir *et al*., 2002) and consequently, the microstructure of fish muscle is affected during chill storage (Loje *et al*., 2007).

The quality and shelf-life of cold-smoking fish is depending on smoking processing, storage conditions (temperature and atmospheres) and physicochemical characteristics of end of product (e.g. WPS, pH, aw) that are used to prediction of

microbiological growth (Dalgaard and Mejlholm, 2019). Food shelf-life extension is important to food manufacturers and freezing and refrigerating conditions are used to food quality and shelf-life extension. Dawson *et al*. (2018) reviewed the effects of freezing on the shelf-life of salmon indicating the extension of shelf-life product and changes on food quality, regardless of the preservation method. Freezing technology can also induce changes in salmon characteristics such as colour, texture, water holding capacity and effects on the fish structure by intracellular/extracellular ice crystal growth (Dawson *et al*., 2018). The use of freezing has also been investigated to control of parasites growth of human health concern (FDA, 2001b).

In cold-smoking industry, the application of a freezing step to raw material, or after cold-smoked fillet salting (before slicing) or, after vacuum packing cold-smoked product before sample commercialisation, were observed in some European smokehouses. This freezing procedure was justified based on the type of smoking process and on market distribution facilities, conditions also observed in Portuguese commercialisation. The effect of freezing on the physicochemical, textural and sensorial characteristics of smoked salmon (*Salmo salar*) has been studied (Martinez *et al*., 2010; Rora and Einen, 2003). However, little information is available about the effects of freezing and storage time on microbiota of vacuum packed cold-smoked fish, before market storage chill conditions. The aim of this study was evaluate the additional freezing step (-20ºC) after vacuum-packaging individual cold-smoked fish samples, as a potential factor to modify the microbial ecology of vacuum-packed cold-smoked salmon-trout (*Oncorhynchus mykiss*) that was previously salted by different procedures (dry and wet for 6h or 4h) and smoked in a pilot-scale industrial smoker. Considering the effects of salting/smoking processing on microstructure of fish muscle, an additional study of myofibrillar proteins using DSC is

presented. DSC is a powerful technique to study the thermodynamics of protein stability and it can provide basic understanding about protein denaturation.

4.2.2 Materials and Methods

4.2.2.1 Fresh fish samples

Fresh salmon-trout (*Oncorhynchus mykiss*) were obtained from supermarkets, originating from Portuguese aquaculture and from the same producer. The samples were shipped in boxes packed with ice in a refrigerated truck and arrived at the laboratory 20 hours after slaughtering. After overnight storage at 5ºC the fish were eviscerated, gutted, filleted and washed in tap water and immediately weighed. The weight average of each eviscerated fish was 1700 g.

4.2.2.2 Salting and smoking procedures

Different combinations of salting and smoking process were performed according to Vaz-Velho (2000). These combinations of treatments were adjusted as follows: a) Dry-salting for 4h and for 6h; b) Wet salting (brine 80º saturation, corresponding at 2100 g salt/L of water) for 4h and for 6h. Dry-salting was performed by adding 1/3 of the weight of fish in the proportion of salt: sugar. A total of eight fillets with weight average of 800$\pm$200 g were placed in a chamber at 8ºC during salting. Draining was done overnight at 5ºC. Smoking was done by drying for 2 hours and smoking for 6 hours. The follow treatments were performed:

Treatment 1: Dry salting during 6h + smoking (2h dry+6h smoke)

Treatment 2: Dry salting during 4h + smoking (2h dry+6h smoke)

Treatment 3: Brining during 6h + smoking (2h dry+6h smoke)

Treatment 4: Brining during 4h + smoking (2h dry+6h smoke)

For that an AGK smoker (Type 135/12, Wallersdorf, Germany) was used. The time/temperature profile of the smoking process was recorded on a portable microprocessor (HI 92804c, Hanna instruments, Portugal). Smoke temperature was kept below 25ºC and the humidity recorded by hygrometer (RotronicAM3, New York, USA) varied from 70% during the first three hours, decreasing to 52% at the end of the smoking process.

The smoked samples were cooled overnight at 5ºC. The following day, lug and pin bones and belly flaps were removed and the fillets were then sliced by hand and vacuum packed in a Multivac-Gastrovac ((Multivac, Germany), 1mbar/10s. The permeability of the packs to O_2, CO_2 and N_2 were respectively 4mol/m^2.d.bar, 13 mol/m^2.d.bar and 4 mol/m2.d.bar.

4.2.2.3 Chilled and frozen samples

A total of 128 vacuum-packed samples were produced (weight of 50g of product) corresponding to 32 samples of each subgroup of treatment previously indicated.

For each one, two different storage conditions was applied: a) stored at 5ºC during 4 weeks and, b) frozen at -20ºC in a freezing chamber (Fitoterm DB 5000 BT, Aralab, Rio de Mouro, Portugal) during 20 days and then stored at 5ºC during 4 weeks. All the samples were analysed weekly for physicochemical and microbiological status during 4 weeks.

4.2.2.4 Microbiological analysis

From each pack. 30 g of cold-smoked fish was taken aseptically into a plastic bag (composite sample of 10g taken from 3 different locations in the vacuum-pack) and homogenised for 90 s in a Stomacher (Seward 400). Ten grams of the mixture were aseptically taken and decimally diluted in sterile Maximum Recovery Diluent (CM 733 Oxoid) and homogenised for 20 s. 'Total viable counts' were performed on spread plates of Long and Hammer's medium (LH) (Van Spreekens, 1974), modified with additional 1% w/v NaCl) and on pour plates of Iron agar Lyngby (IR) (Gram *et al*., 1987) incubated aerobically at 20 ºC for 5-7 days and 21ºC for 3 days, respectively. Counts of lactic acid bacteria (LAB) were made in pour plates of Nitrite Actidione Polymyxin agar aerobic plate (NAP) medium pH 6.7 (Davidson & Cronin, 1973) incubated anaerobically at 21ºC for 5 days. *Enterobacteriaceae* counts were made in pour plates of 5 ml Violet Red Bile Glucose Agar (VRBGA) which after 2 h at 20-25 ºC were overlaid with 15 ml of the same medium. Typical *Enterobacteriaceae* colonies were counted after 2 days of incubation at 30 ºC. To assess the selectivity of the different media, representative colonies were picked from the plates and the following tests were performed: cell morphology, Gram stain, catalase and oxidase tests.

4.2.2.5 Physicochemical analyses

The NaCl and moisture content of the finished products were evaluated according AOAC (1995), method 937.09 and method 24.003, respectively. Salt content in water phase (WPS) was calculated as described by Huss *et al*., (1997), from the total salt and water contents, using the equation: SWP = % salt / (% salt + % moisture) * 100. Water

activity (a_w value) was calculated from the corresponding SWP value, using the appropriate equation (Gimenéz and Dalgaard, 2004):

$a_w = 1 – 0.0052471 \times WPS – 0.00012206 \times WPS^2$.

The pH was measured in smoked fish homogenates (5g of fillet homogenised with 5 ml of deionized water. stomached for 2 min) with a micropH 2002 pH meter (Crison, Barcelona, Spain).

4.2.2.6 Differential Scanning Calorimetry

DSC has been used to study the thermal properties of fish muscle proteins and to measure the extent of denaturation under various processing conditions (Thorarinsdottir *et al*., 2002). Proteins may be analysed in situ without solubilisation of the muscle tissue. The DSC study was performed on Shimadzu Corporation TA-50 DSC System (Shimadzu, New York, USA). The instrument was temperature-calibrated, using water and indium. Enthalpy was calibrated with indium. Empty pans were used as reference. Slices, free of connective tissue, were dissected from whole muscle. Samples, weighing 20 mg (accuracy of 0.01 mg) were sealed in Shimadzu volatile sample pans and scanned at a heating rate of 10ºC/min over the range 15– 110ºC.

4.2.2.7 Statistical analysis

Analysis of variance was carried out for microbiological and physicochemical composition of vacuum-packed cold-smoked salmon-trout samples that were treated by different salting/smoking with and without addition freezing step were performed by ANOVA, using a statistical program IBM SPSS® Statistic 23.0 for Windows® (2015, SPSS Inc., Chicago, USA). Separate univariate analyses were performed for each response variable. The salting method (dry or wet salting), salting time (6h or 8h) in addition to

freezing step were entered as treatment factors into multivariate models. Storage time (1, 2, 3, and 4 weeks) was used as a covariate in the model when analysing parameters. Data presented in figures and tables are mean values based on those effects at significance level of 5%.

4.2.3 Results and Discussion

4.2.3.1 Physicochemical characteristics of cold-smoked salmon-trout fillets

The characteristics of cold-smoked fillets previously treated by dry salting or brining followed by drying/smoking are presented in Table 1. The results showed a significantly low weight loss for samples in which brine was carried out for 4 hours in comparison with other type/time of salting fillets studied ($p<0.05$). The weight loss is directly related to the size of the fish (or fillet) and with the type and time of the curing process. An increase of weight loss was expected from the process involving dry salting, together with longer periods, in agreement with the results presented. Similar results were obtained by Sigurgisladottir *et al*. (2000) reporting significant lower yield for drysalting cold-smoked fillets comparing with brining process. However, the homogeneous distribution of salt during dry salting (as compared with brining) seems to be difficult to attain and could be a factor with influence on the quality of salted fillets. Therefore, differences on microbiological counts can be observed in difference pieces/slices of the same fillet. In this study, the WPS did not achieve the safe limits for these products (>3.5% w/v) in all cold-smoked samples, according to the recommendation of FDA (2001a). However, dry or wet salted samples did not show significant differences in WPS nor in a_w

(p>0.05). FDA (2015) reported that in a commercial operation with a target of 3.5% of WPS, the final products ranged from 2.8% to 6.0 % with the majority of the samples being above 4.0%. An average of salt content of 3.1$^{\pm}$ 0.6% (w/w) on cold-smoked fish was showed by Cardinal *et al*. (2004), with significant differences depending on the country of production. In fact, the variability of WPS in products at the end of the salting/smoking process can impose serious problems of safety, and storage controlled conditions should be imposed regarding the storage temperature and type of packaging (FDA, 2015).

Table 1. Overall results for physicochemical status of cold-smoked fillets

Treatments	Salted process	Salted/Smoked fish Fillets	Weight loss (%)(**)	WPS (**)	aW (**)
Treatment1	Drysalting, 6h	A / B	27.86±5.13a	3.15±0.64a	0.982+0.003a
Treatment2	Drysalting, 4h	C / D	28.02±5.06a	2.30±0.28a	0.987+0.001a
Treatment3	Brining, 6h	E / F	24.78±0.74a	2.75±0.64a	0.985+0.003a
Treatment4	Brining, 4h	G / H	8.39±1.07b	1.80±0.28a	0.990+0.001a

(*) Columns with different digit differ statistically (p<0.05); (**) Numbers are average of two fillets samples.

4.2.3.2 DSC thermograms of myofibrillar proteins during salting/smoking processing and storage

Changes in DSC thermograms of salmon-trout myofibrillar muscle proteins during cold-smoking processing (fresh fish, salting and smoking) and during chill storage are shown in figure 1. The myofibrillar proteins in fresh salmon trout are represented by a

large peak of myosin (Tmáx 46ºC) and actin (Tmáx 77ºC) and other peaks less representative, corresponding to sarcoplasmic proteins. Thorarinsdottira *et al*. (2002) reported similar patterns of muscle proteins in other fish species. Due to the loss of water and salt-soluble proteins during cold-smoked processing, changes in myofibrillar proteins were observed (Figure 1a). The commonly observed loss in weight during cold-smoked fish process, is due to dehydration of the muscle and to lipids leaching from the muscle, which correspond to a total of 10-25%, depending on the raw material and the final product characteristics (Sigurgisladottir *et al*., 2000). During chill storage, Loje *et al*. (2007) reported that the initial smoked fish had a higher liquid holding capacity (LHC) than samples stored for 20 days and the changes were consistent with the change in water distribution, which could indicate denaturation of muscle proteins. Thereby, changes in texture of salmon trout can occur and may result in difficulties in slicing operation of smoked fillets. This justifies the inclusion of a freezing step after smoking and before slicing of smoked fillets by some cold-smoke fish producers.

Sigurgisladottir *et al*. (2000) also showed differences in fiber diameters in function of raw fish origin and type of salting applied. The same authors showed that the ocean-salmon presents small fiber diameters and higher shear force when compared to farmed salmon. Also, a small cross-sectional area of fibers was observed in dry salted fillets (fibers shrunk more during drysalting) than in brine salted fillets. The interaction of raw material characteristics and smoking processes on effects of quality of smoked salmon has been studied (Rora and Einen, 2003; Sigurgisladottir *et al*. 2001). Interesting results were obtained by Sigurgisladottir *et al*. (2000) and concluded that salt penetrate better in fish previously frozen as the cells structure have been changed during freezing, causing an increase of salt diffusion into the muscle, and consequently a higher percentage of SWP.

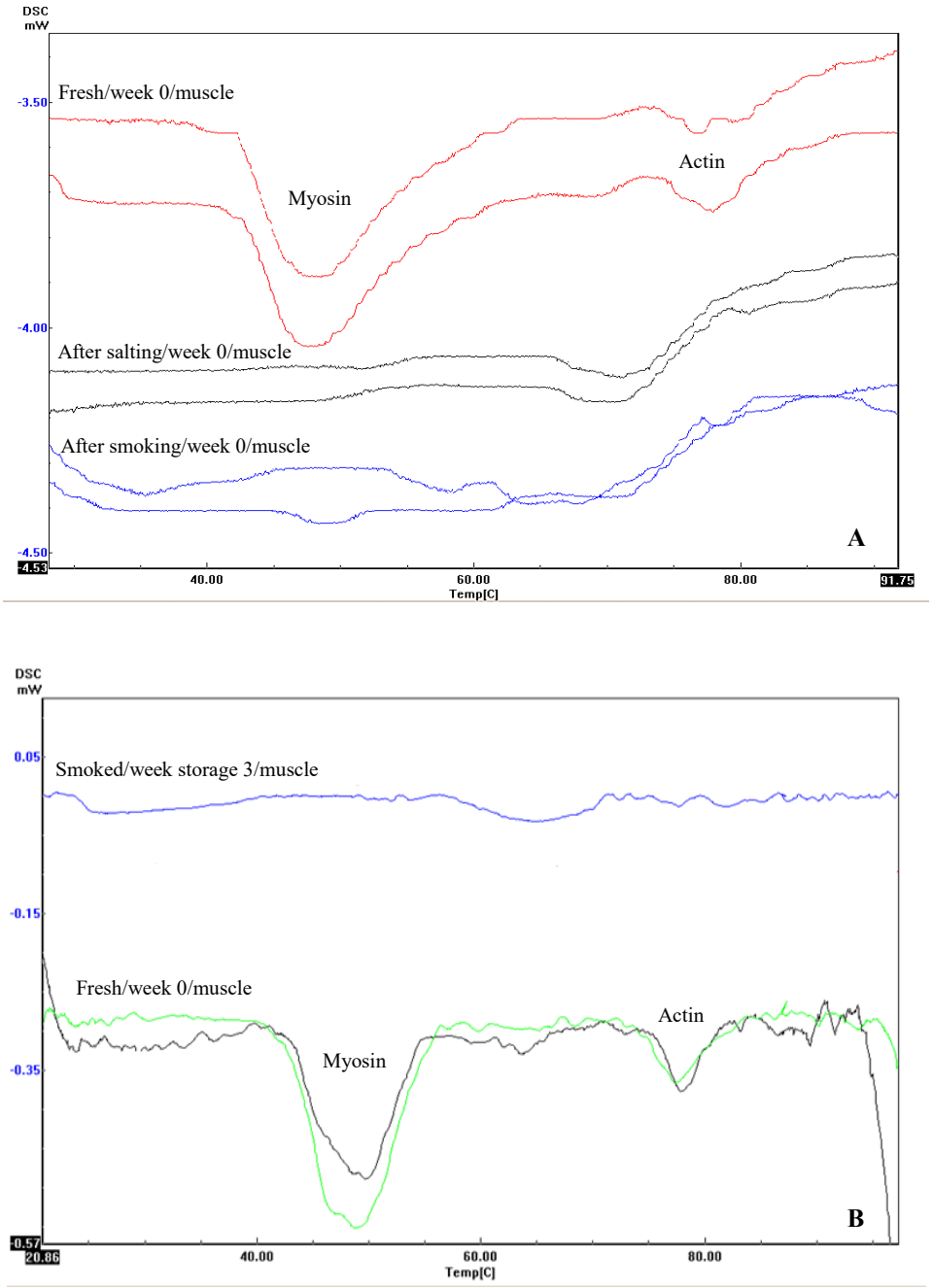

Figure 1. Comparison of different DSC thermograms of myofibrillar proteins for fresh salmon trout and during processing (after salting and smoking) (A) and during chill storage (B) of cold-smoked salmon trout.

In this study, the salting/smoking process led to some shifts in transition temperatures as well as a decrease in peak area (Figure 1a). The peaks became lower broader and less separable, compared to the thermogram of the fresh salmon trout, showing difficulty to measure using DSC. Similar thermograms were showed for cold-smoked salmon trout during chill storage (Figure 1b).

4.2.3.3 Microbial ecology of vacuum-packed cold-smoked salmon-trout

Microbiological evolution during chill storage of cold-smoked samples are shown in Figure 2, for samples with and without a previous freezing step (-20ºC) and subsequently stored at 5 ° C. After 3 weeks of chill storage, the numbers of APC, LAB, H_2S-producing bacteria and *Enterobacteriaceae*, reached to 10^5-10^7 cfu /g, 10^3-10^5 cfu /g, 10-10^4 cfu /g and 10-10^2 cfu /g, respectively, for unfrozen samples (Figure 2-A1, B1, C1 and D1). The levels are related to salted process, and being in agreement within the results previously published by other authors (Espe *et al*., 2004; Leroi *et al*., 2000; Silva and Gibbs, 2015). Increases in levels of different microorganisms were observed for frozen, 10^6-10^8 cfu /g for APC, 10^3-10^6 cfu /g for LAB, 10^3-10^6 cfu /g for H_2S-producing bacteria and 10 -10^3 cfu /g for *Enterobacteriaceae* (Figure 2 -A2, B2, C2 and D2). Higher microbiological counts at first week of chill storage were observed for APC, LAB and H_2S-producing bacteria (up to 1Log_{10} UFC/g) in samples submitted to a freezing process. This could be attributed to a high moisture and nutrient content of samples, promoting the growth of microorganisms. Changes in storage temperature of frozen samples (from frozen to chilled conditions) occurred in this period. Freezing is a good preservation method to control the microbial growth in fish, however, it induces physicochemical changes during storage (Kilibarda *et al*., 2009). Results indicated that a previous step of freezing had

influence on microbial ecology of chilled cold-smoked salmon trout ($p<0.05$), with exception of *Enterobacteriaceae* group ($p>0.05$) (Table 2). Considering the samples previously frozen, H_2S-producing bacteria showed the major differences during chill storage and for all type of salted samples (dry or wet, 6h or 4h) ($p<0.05$) (Table 3). This group of microorganisms were influenced by combined effect of salted/smoked and freezing conditions (Table 2 and 3).

This group of microorganisms belongs to spoilage bacteria associated to fish, such as *Sh. putrefaciens*, *Aeromonas* spp. and *Ph. phosphoreum* (Dalgaard, 2000; Gram *et al*., 2002; Leroi, 2011). *Sh. putrefaciens,* which is capable of anaerobic respiration, can be selected by vacuum packaging and for the CO2-resistant, psychrotolerant marine bacterium *Ph. phosphoreum*. However, none of these three bacteria when inoculated in cold-smoked salmon caused spoilage of vacuum-packed cold-smoked salmon, whereas the co-innoculation studies with *B. thermosphacta* and *Carnobacterium piscicola* produced spoilage odours (Joffraud *et al*., 2001). Jorgensen *et al*. (2000b) described the term 'metabiotic spoilage association', where two or more microbial species contribute to spoilage, through exchange of metabolites or nutrients, that could be covered by the 'specific spoilage organisms' concept, where a consortium of organisms interact to spoil the product. Recent studies reported the importance of synergistic effects of physicochemical parameters on microbiological growth of cold-smoked fish (Iacumin *et al*., 2017; Lerfall and Osterlie, 2013; Martinez *et al*., 2013).

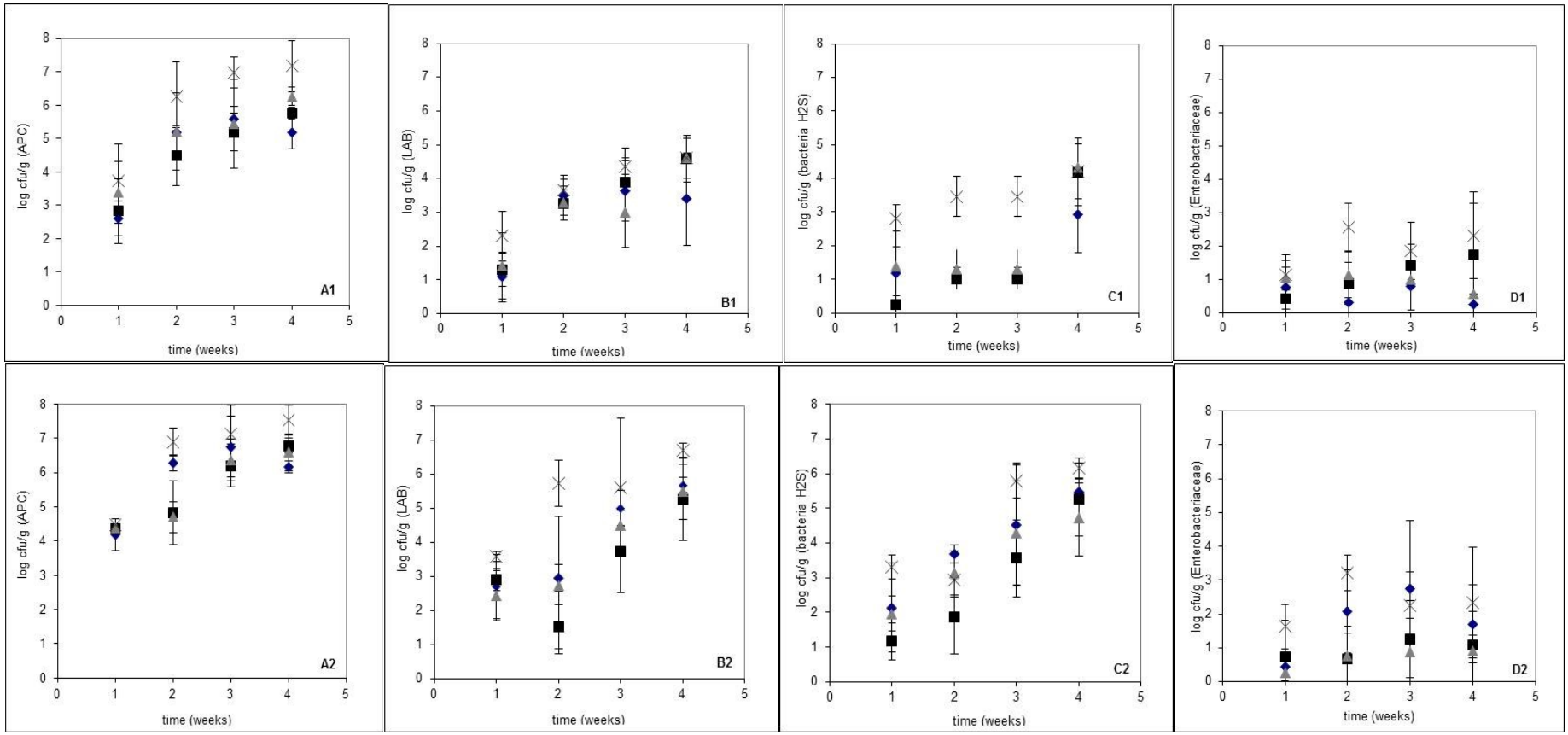

Figure 2. – Microbiological evolution (APC- Aerobic Plate Count; LAB; H_2S- producing bacteria; Enterobacteriaceae) of vacuum-packed cold-smoked salmon-trout during chill storage in function of salting process (Δ Drysalting, 6h; ■-Drysalting, 4h; ♦-Brining, 6h; x-Brining, 4h). (A1, B1, C1, D1) – cold-smoked samples stored at chill conditions; (A2, B2, C2, D2) – cold-smoked samples previously freezing at (-20ºC).

Table 2. Overall results for pH and microbiological characteristics of vacuum-packed cold-smoked salmon-trout with (P2) and without (P1) a previous freezing step before storage at chill conditions (5ºC)

	Salted process	N	pH (**)		APC (*)		LAB (*)		Enterobacteriaceae (*)		H_2S- producing bacteria (*)	
			P1	P2	P1	P2	P1	P2	P1	P2	P1	P2
Treatment1	Drysalting, 6h	16	6.06±0.09a	5.87±0.12cd	4.64±1.46a	5.84±1.11abcd	2.90±1.37a	4.08±1.42b	0.54±0.65a	1.74±1.47bcd	1.58±1.01a	3.96±1.61cd
Treatment2	Drysalting, 4h	16	5.97±0.06ab	5.88±0.18cd	4.57±1.40a	5.54±1.13abc	3.26±1.41ab	3.35±1.63ab	1.13±0.96abc	0.93±0.83ab	1.60±1.64a	2.96±1.85b
Treatment3	Brining, 6h	16	5.89±0.09bcd	5.95±0.15bc	5.08±1.29ab	5.50±1.09abc	3.08±1.37a	3.78±1.65ab	0.95±0.65abc	0.71±0.87a	2.08±1.51a	3.53±1.44bc
Treatment4	Brining, 4h	16	5.95±0.14bc	5.83±0.26d	6.04±1.49cd	6.50±1.31d	3.73±1.03ab	5.40±1.51c	1.97±0.97cd	2.35±1.11d	3.48±0.76bc	4.55±1.53d

(P1) Process without a previously freezing step before chill commercialization; (P2) Process with a previously freezing step at (-20ºC) before chill commercialization.

(WPS) Water Phase Salt, measure in percentage.

(*) Microbiological values are mean of sixteen samples for each process (P1 or P2) and represent the CFU/g of sample. Columns with the different digit differ statistically ($p<0.05$).

(**) Values of pH are de mean of two samples

Table 3 Results from analysis of variance of the data concerning individual and combined treatments of cold-smoking salmon trout

Parameters	Freezing step (Fstep)	Salting method (Smethod)	Time Salting (Tsalting)	Interactions		
				Fstep x Smethod	Smethod x Tsalting	Tsalting x Smethod x Tsalting
Weight Loss (WL)	-	<0.05	<0.05	-	<0.05	-
Water Phase Salt (WPS)	-	ns	ns	-	ns	-
a_W	-	ns	ns	-	ns	-
pH	<0.05	ns	<0.05	ns	<0.05	ns
APC (Aerobic Plate Count)	<0.05	<0.05	<0.05	ns	<0.05	ns
LAB (Lactic Acid Bacteria)	<0.05	<0.05	<0.05	<0.05	ns	ns
H_2S producing bacteria	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Enterobacteriaceae	ns	<0.05	<0.05	<0.05	ns	ns

Significance level of 5%.

In this study, different salted samples revealed differences on physicochemical characteristics and on microbiological patterns (Table 2). The additional step of freezing resulted on significant increase of LAB, H_2S-producing bacteria and *Enterobacteriaceae* ($p<0.05$), and a decrease in pH ($p<0.05$) (Table 2), especially for dry salted samples (6h) (treatment 1). As referred by Barat *et al*. (2002) the prior freezing can induce changes in the structure of the product related to the state of the proteins in fish muscle. Thorarinsdottira *et al*. (2001) indicated that the salt concentration affects the stability and denaturation of proteins and thereby physicochemical factors such as water holding capacity (WHC), resulting in swelling. Maximum swelling has been estimated at approximately 5% of salt but, at higher concentrations, the myofibrilar proteins rapidly lose water through the salting-out process (Honikel, 1989). In this study, the yield was dependant on type of salting/smoking, showing brining process for short time (4h) the lower weight loss ($p<0.05$), lower percentage of WPS, without significant influence on a_w ($p>0.05$) (Table 3). The salt concentration and temperature in the process are both essential parameters to control the microbial growth. The selective 'pressure' by addition of salt and drying/smoking of salmon trout, switches the microbiota in the same direction as vacuum-packed products, thus the LAB, *Enterobacteriaceae* and some *Brochotrix* can be increased (Gram *et al*., 2001), as well as other microorganisms depending on raw material freshness and the environmental contamination level.

Freezing before or after salting/smoking fillets can affect the fish muscle structure. Freezing before smoking results on increase of product yield and water content, however softer texture and gaping can be observed (Rora and Einen, 2003). The same authors appointed that freezing only after smoking led to fewer changes in fish quality. These results were similar to those presented by Martinez *et al*. (2010) regarding to salting/smoking and freezing at -25ºC for 24h and maintaining the product at -18ºC,

increased the shelf-life of product (>45 days). On the other hand, Kilibarda *et al*. (2009) showed high water and NaCl, as well SWP in the vacuum-packed smoked fillets produced from frozen fish, and as well as total number of bacteria and lactobacilli, statistically different from samples produced from fresh fish. Beaufort *et al*. (2009) reported an innovative freezing technological process, named 'superchilled' storage (-2ºC for 28 d) on cold-smoked salmon, which consists on storing food just above the initial freezing temperature (-2ºC/-3ºC) and also depending on salt content. The same authors referred to a limited impact on some of the organoleptic properties of cold-smoked salmon but the level of *L. monocytogenes* at the end of the shelf-life (4ºC for 10d and 8ºC for 18d) could exceed the microbiological criterion set by European legislation.

The effects of temperature as an additional step of storage needs to be carefully applied, since the frozen step can induce changes on the structure of the product, resulting in changes of microbiological ecology. Dawson *et al*. (2018) mentioned the denaturation of the protein is one problem caused by slow freezing with protein denaturation-dependent upon temperature. The application of additional conservation processes does not preclude the possibility of including these in the commercial process which should be studied by checking their relevance, achieving product stabilisation and safety.

4.2.4 Conclusions

The application of a previous freezing step (-20 ºC) after vacuum-packaging of cold-smoked salmon-trout (*Oncorhynchus mykiss*) resulted in changes on the microbial ecology. A general increase in microbiological numbers in vacuum-packed cold-smoked samples previously frozen and at initial stage of chill storage, was observed for APC, LAB

and H_2S-producing bacteria, with particular relevance for this last group of bacteria during storage.

The salting/smoking process significantly decreased the heat stabilities of both myosin and actin contributing to structure instability of the muscle. The additional freezing step after salting/smoking salmon trout fillets can also have influenced the fish muscle structure. The effect of additional freezing on microbiological growth in cold-smoked salmon trout samples was dependent on the type of salting process applied. The potential growth of spoilage microorganisms and others, e.g. pathogens during chill storage, indicate that the application of the frozen process should be studied (as temperature *vs* time) by checking their efficacy on industrial/traditional production of cold-smoked fish, considering the critical control of point related to storage temperature and WPS.

4.3 An assessment of the processing and physicochemical factors contributing to microbiological selection in cold-smoked salmon trout (*Oncorhynchus mykiss*) stored in vacuum and modified atmosphere packaging

Abstract

The effect of different combined treatments of salting/drying/cold-smoking on microbiological and physicochemical characteristics of salmon trout fillets (*Oncorhynchus mykiss*) packed under vacuum (VP) and modified atmosphere (MAP) conditions) was evaluated during 5 weeks of storage at 5ºC. The tested conditions were as follows: (dry or wet salting, 8h or 6h with different proportions of salt:sugar|3:1 or salt:sugar|5:1) and smoking (short or long smoke exposure) in combination with VP or MAP. In general, brine-salted fillets showed lower percentages of weight loss and water phase salt content (WPS) compared to dry-salted fillets. The different proportions of sugar in the salting process seems not to induce significant differences in the physicochemical parameters of cold-smoked fillets at the end of smoking process ($p>0.05$), however, it was clearly demonstrated that samples with higher sugar content in the salting mixture (salt:sugar|3:1) induced an increase in microbiological numbers. Overall, dry salting is preferable to brining for reducing, microbial growth in cold-smoked salmon trout stored in VP. The smoking process characterized by long drying and short smoking times (Group II – Dry 6h and Smoke 2h) encouraged a general increase in microbiological numbers of studied samples, with a positive LAB increase, but a negative effect on the samples' to microbiological quality, with a significant increase in *Enterobacteriaceae* and H_2S-producing bacteria. In this group of samples, significant levels of trimethylamine (TMA) (up to 30 mg. in 100 g of fish) during chilled

storage were observed. This result was supported by 48.9% of bacteria positive for TMA production when tested in culture medium and isolated from VP (Group II-VP) and an increase to 70.1% in samples isolated from MAP (Group II- MAP). A positive effect of smoke exposure on microbial ecology was observed in Group I dry-salted samples packaged either in VP or MAP. Despite an increase in Aerobic Plate Counts (APC) and H_2S-producing bacteria after 3 weeks of storage in MAP (Group I- MAP), a decrease in *Enterobacteriaceae* numbers and a small increase in LAB counts were observed.

4.3.1 Introduction

Smoking is an ancient process for preserving and flavouring food. The original principle of preservation was due to a combination of lower water activity in the product and the uptake of smoke's bactericidal and antioxidant components by the product, adding sensory characteristics such as a smoky colour and flavour to the product. There are three stages of cold-smoke processing that contribute to the preservative effect: salting, dehydration and smoking, which together modify the development of chemical and microbial properties (Lovdal, 2015). The mild temperatures applied (20-30ºC) do not kill the microbiota and the absence of a thermal treatment makes the parameters of salting and drying/smoking crucial to achieving the ideal water phase salt (WPS) level and minimize the risk of foodborne hazards and spoilage processes. Salt can be applied in dry form, as brine, or by the high-pressure injection of brine (Birkeland and Bjerkeng, 2005). According to Huss (1994), cold-smoked fish can be considered "lightly preserved fish" products with a salt content of <6% NaCl (w/w) in water phase and low acid content (pH>5.0), having a limited shelf life, depending on storage

temperature (FDA, 2001). Different shelf lives are commonly found in the marketed cold-smoked fish products meaning these products have different compositions regarding microbiological characteristics and product stabilization.

In past years, attention has been focused on characterizing the dominant microbiota present in cold-smoked salmon during chilled storage and their interactions in relation to spoilage characteristics and safety (Leroi, *et al*., 2000; Jorgensen, 2000; Leroi *et al*., 2001). On the other hand, there are a few studies on salmon-trout microbiota (Iacumin *et al*., 2017; Jittinandana *et al*., 2006; Silva and Gibbs, 2015) The role of LAB in lightly preserved food products (LPFP) has not been very thoroughly characterized, but some studies link LAB and Gram negative microorganisms to the spoilage of cold-smoked fish (Palludan-Muller *et al*., 1998; Huss, 1995; Joffraud *et al*., 2006). In fact, health problems for consumers are related to the presence of *Listeria monocytogenes,* which grows very well in different kinds of cold-smoked fish because of its resistance to parameters such as temperature, saltiness/dryness and smoke phenols (Lovdal, 2015). Modified Atmosphere Packaging (MAP) has been found to be efficient in prolonging the shelf life of perishable products, whether raw or minimally processed, such as CSS (Sandhya, 2010). The antimicrobial effect of MAP, mainly caused by carbon dioxide, is limited and combining it with other treatments must be considered in order to enhance its preservative role in perishable foods (Farber, 1991).

The use of a controlled technological process plays an important role in the selection of microbial ecology. The best combined treatments in a core process should not only inhibit/restrict the numbers of microorganisms, but also provide conditions for the selection of an appropriate microflora. Lactic Acid Bacteria (LAB) have been indicated as dominant microflora with potential to spoil vacuum-packaged cold-smoked fish (Paludan-Müller *et al*., 1998; Lyhs *et al*., 1999; Rodríguez *et al*., 2002; Silva *et al*.,

2002; Cardinal *et al*., 2004; Tomé *et al*., 2008; Silva and Gibbs, 2015). Many attempts to produce a naturally safe, cold-smoked fish product using LAB as biopreservation have been studied by different authors (Calo-Mata *et al*., 2008; Leroi, 2018; Gahnbari *et al.*, 2013; Tomé *et al*., 2009, 2010) for the same type of products.

Different combinations of salting/drying/smoking treatments in conjunction with vacuum packing (VP) or modified atmosphere packaging (MAP) were studied in order to ascertain influence of such production processes on the level and types of bacteria and physicochemical properties found in cold-smoked salmon trout products (*Oncorhynchus mykiss*) during 5 weeks storage at 5ºC.

4.3.2 Materials and Methods

4.3.2.1 Fish samples and treatments

Fresh salmon trout (*Oncorhynchus mykiss*) weighing 2.50±0.25 Kg were obtained from a fish farm in Pisões, in Northeast Portugal. The fish were shipped in boxes packed with ice in a refrigerated truck and arrived at the laboratory 12 hours after slaughter. After overnight storage at 5ºC, the fish were eviscerated, gutted, filleted and washed with tap water.

Two groups of eight salmon trout each were prepared.8% over the salting period. The fillets were salted using two processes:

(1) dry salting, 1/3 of the fish weight was calculated as a reference for total weight of the salt/sugar mixture and added in the proportions of 3/4 salt:1/4 sugar (1st combination) and 5/6 salt:1/6 sugar (2nd combination);

(2) wet salting, the same proportions were used for the brine solution (80º brine, which corresponds to 2.11 Kg/L of salt): 158 Kg salt: 0.53 Kg sugar (1st combination) and 1.76 Kg salt and 0.35 Kg sugar (2nd combination).

During salting, the fillets were placed in a chamber at 16ºC. Draining was done overnight at 5ºC. The different treatments combinations are shown in Table 1.

Drying/smoking was carried out using two different processes (Group I and II). Group I, drying for 2 hours and smoking for 6 hours, and Group II, drying for 6 hours and smoking for 2 hours. In total, 32 fish fillets were smoked using the two processes. An AGK smoker (Type 135/12, Wallersdorf, Germany) was used. A more sensitive thermostat and timer (lae® electronic, MTR12, Italy) was coupled to the smoker. Smoke was produced by smouldering beech wood chips (Räucher Gold®, type Kl 2/16, Germany). The time/temperature profiles of the two processes were recorded on a portable microprocessor (Hanna instruments, HI 92804c, Portugal). The smoke temperature was kept below 30ºC in the two groups of experiments (Group I: 2 hours of drying + 6 hours of smoking; Group II: 6 hours of drying + 2 hours of smoking). Humidity, recorded by an hygrometer (Rotronic AM3, New York, USA) varied between 70% during the first three hours and decreasing to 52% at the end of the smoking process.

The smoked samples were cooled overnight at 5 ºC. The following day, lug and pin bones and belly flaps were removed and the fillets were then sliced by hand and packed in vacuum and in MAP conditions with a mixture of CO2|N2 (60%|40%) using a Multivac-Gastrovac (Multivac, Germany) at 1mbar/10s. The permeability of the packs to O_2, CO_2 and N_2 was 4mol/m^2.d.bar., 13 mol/m^2.d.bar and 4 mol/m^2.d.bar respectively.

The packs were stored for 5 weeks at 5ºC for weekly analysis. Sampling was performed weekly, by using two bag samples of each group for the chemical and microbiological analyses.

Table 1 - Experimental design for salting/drying/smoking of salmon-trout packed in VP and MAP

Group I		Group II	
8 fish | 16 fillets Combined salting/drying/smoking treatments		8 fish | 16 fillets Combined salting/drying/smoking treatments	
Step 1: Salting process 2 pair of fillets- dry salting for 8 h (1/4 sugar) 2 pair of fillets- dry salting for 6 h (1/4 sugar) 2 pair of fillets- brining for 8 h (1/4 sugar) 2 pair of fillets- brining for 6 h (1/4 sugar) 2 pair of fillets- dry salting for 8 h (1/6 sugar) 2 pair of fillets- dry salting for 6 h (1/6 sugar) 2 pair of fillets- brining for 8 h (1/6 sugar) 2 pair of fillets- brining for 6 h (1/6 sugar) Step 2: Drying/Smoking process Combination: 2h drying + 6h smoking		Step 1: Salting process 2 pair of fillets- dry salting for 8 h (1/4 sugar) 2 pair of fillets- dry salting for 6 h (1/4 sugar) 2 pair of fillets- brining for 8 h (1/4 sugar) 2 pair of fillets- brining for 6 h (1/4 sugar) 2 pair of fillets- dry salting for 8 h (1/6 sugar) 2 pair of fillets- dry salting for 6 h (1/6 sugar) 2 pair of fillets- brining for 8 h (1/6 sugar) 2 pair of fillets- brining for 6 h (1/6 sugar) Step 2: Drying/Smoking process Combination: 6h drying + 2h smoking	
Step 3: Packaging VP or MAP (CO2|N2, 60%|40%)		Step 3: Packaging VP or MAP (CO2|N2, 60%|40%)	
Group I-VP Group Ia (Dry salting -VP) Group Ib (Dry salting -VP) Group Ic (Brining -VP) Group Id (Brining -VP) (80 samples packed)	Group I- MAP Group Ia (Dry salting – MAP) Group Ib (Dry salting – MAP) Group Ic (Brining – MAP) Group Id (Brining – MAP) (80 samples packed)	Group I-VP Group Ia (Dry salting -VP) Group Ib (Dry salting -VP) Group Ic (Brining -VP) Group Id (Brining -VP) (80 samples packed)	Group I- MAP Group Ia (Dry salting – MAP) Group Ib (Dry salting – MAP) Group Ic (Brining – MAP) Group Id (Brining – MAP) (80 samples packed)
Step4: Storage in chilled conditions for 5 weeks (5ºC)		Step4: Storage in chilled conditions for 5 weeks (5ªC)	

4.3.2.2 Microbiological and physicochemical analysis

Ten grams of cold-smoked salmon trout samples were taken aseptically from each bag, homogenized in 90 ml of ¼ strength Ringer's solution for 2 min in a Stomacher 400 LAB Blender (Seward Medical, London, England). Serial decimal dilutions in ¼ strength Ringer's solution were prepared.

Aerobic Plate Counts (APC) were performed by spread plating suitable dilutions on Long and Hammer's medium (LH) (L & H, as modified by Van Spreekens, 1974) with an additional 1% w/v of NaCl (Merck, Darmstadt, Germany) and on iron agar Lyngby (IR) pour plates (Gram *et al*., 1987), incubated aerobically at 20ºC for 5-7 days and 21ºC for 3 days respectively. Lactic acid bacteria (LAB) were enumerated by pour plating in Nitrate Actidione Polymyxin (NAP) agar medium, pH 6.7 (Davidson & Cronin, 1973), incubated anaerobically at 21ºC for 5 days. *Enterobacteriaceae* were listed using overlaid plates of Violet Red Bile Glucose Agar (VRBGA, Biokar Diagnosis, Beauvais, France) in pour plates of 5 ml VRBGA, which after 2 h at 20-25ºC were overlaid with 15 ml of the same medium. Typical *Enterobacteriaceae* colonies were counted after 2 days of incubation at 30ºC. To assess the selectivity of the different media, representative colonies were picked from the plates and the following tests were performed: cell morphology, Gram stain, catalase and oxidase tests.

Representative colonies from different growth medium plates were selected and examined for reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA), measured by a pH change in the cultured medium (Maille and Tailliez, 1986). Proteolytic and lipolytic bacteria activity was performed on milk agar (10%) (Sigma-Aldrich) and tributyrin agar (Merck, Darmstadt, Germany), respectively. Selected colonies were spread on differential culture media and, after incubation, the positive reaction was interpreted by the presence of a translucent halo surrounding the colonies.

Representative colonies from VRBGA plates were selected and examined to genera identification using EPM-medium (Toledo *et al*., 1982a), MILi medium (Toledo *et al*., 1982b) and citrate test (Holt and Krieg, 1984).

The salt concentration was determined according to AOAC (1995) method 937.09. Salt content was expressed as % of NaCl in water phase of muscle, calculated from the corresponding total salt and water contents, using the equation:

SWP = % salt ×100/ (% salt + % water).

The moisture contents of the finished products were determined according to AOAC (1995) method 24.003. Water activity (a_w value) was calculated from the corresponding SWP value, using the equation (Gimenéz and Dalgaard, 2004):

$a_w = 1 – 0.0052471 \times WPS – 0.00012206 \times WPS^2$.

The pH was measured in smoked fish homogenates (1:1) by mixing 5g of fillet homogenised with 5 ml of deionized water and stomached for 2 min using a micro pH 2002 pH meter (Crison Barcelona, Spain).

4.3.2.3 Trimethylamine (TMA) determination

A gas chromatography (GC) method developed using Equilibrium Vapour Analysis (EVA) was used to detect the presence of TMA in the headspace of an enclosed vial. To determine the TMA production in the smoked fish samples, 10 g of each sample was homogenised in stomacher bag with 12 ml of 0.6N perchloric acid followed by centrifugation for 10 min at 3000 rpm, and the supernatant was filtered using Whatman Nº1 paper. The filtrate collected was centrifuged under the same conditions and filtered again. The filtrates were combined and the volume was made up to 25 ml with perchloric acid (0.6 N). 1 ml of the sample volume was added to a vial, and 0.2 ml of internal standard of n-propylamine (2%) added. 1 ml of KOH (65%) was then added and the closed vials were heated to 60 ºC for 10 min. After that a 0.2 ml

volume of headspace was analysed by GC (Perkin Elmer Autosystem, USA); separations were done using a 25m*0.32mm PoraPLOT Amines column Agilent J&W. The GC was carried out in the followed conditions: Carrier gas pressure (Helium, 100Kpaand flow of 1ml/min); Injector temperature (200ºC); Detector (FID) temperature (200ºC) FID hydrogen flow (45 ml/min); FID air flow (450 ml/min); The column oven temperature was: Initial temperature (100ºC); Initial time (3 min); Rate 1 (100ºC to 200ºC at 8ºC/min); Final hold time (15 minutes); The results were expressed as mg of TMA /100 g of fish.

4.3.2.4 Statistical analysis

Analysis of variance was carried out for microbiological and physicochemical composition of vacuum-packed cold-smoked salmon-trout samples that were treated by different salting/smoking/packaging were analysed by ANOVA multifactorial using a statistical program IBM SPSS® Statistic 23.0 for Windows® (2015, SPSS Inc., Chicago, USA). The salting method (dry or wet salting), salting time (6h or 8h), smoking (short dry 2h and long smoke 6h or long dry 6h and short smoke 2h) and packaging (vacuum or modified atmospheres) were entered as treatment factors into factorial models. Separate univariate analyses were performed for each response variable. Storage time (1, 2, 3, and 4 weeks) was used as a covariate in the model when analysing parameters. Data presented in figures and tables are mean values based on those effects at significance level of 5%. For effects of sugar concentration in salting mixture, analysed using one-way ANOVA were performed.

4.3.3. Results and discussion

4.3.3.1 Physicochemical characteristics of salted/dried/smoked salmon trout fillets

The physicochemical characteristics of cold-smoked fillets salted using different proportions of salt:sugar and different types of salting (dry or wet) are shown in Table 2 and Table 3 respectively. Not excluding the possibility that the presence of sugar modifies the microbial ecology of packed salmon-trout samples during chilled storage (discussed in next section), the presence of different proportions of sugar in the salting process does not seem to induce significant differences in the physicochemical parameters of cold-smoked fillets at the end of the smoking process ($p>0.05$) (Table 2). However, samples with a high proportion of salt (in relation to sugar) (salt:sugar|5/6:1/6) showed a higher WPS percentage. Samples salted using dry or wet processes showed significant differences in weight loss ($p<0.05$) (Table 3). In general, brine-salted fillets show lower percentages of weight loss and water phase salt contents (WPS) compared to dry-salted fillets. In the brining process the fish is immersed in the brine and the distribution of salt on the fillets is more uniform compared to dry salting method (Erkmen, 2012). Salt absorption by the fish is slower and lower than in dry salting because the salt is absorbed by the fish as the fish discharges its water into the brine (Tunalı, 1984). Consequently, fish salted by brining contains a higher amount of water and decomposes more quickly than fish salted by dry salting, and its storage life is normally shorter (Erkmen, 2012). This process may be favourable for some fish products once there is less contact with oxygen, which consequently can prevent oxidative flavours with only a slight increase in a_w.

Table 2 – Characteristics of salted cold-smoked fillets according to different proportion of sugar in the salting process

	Salt:Sugar proportion	Salted fillets (N)	Mean (Sd)	Level of significance
Weight Loss (%)	(3/4:1/4)	n=16	9.12 (2.05)	p>0.05 (0.363)
	(5/6:1/6)	n=16	8.39 (2.41)	
NaCl in WP (%)	(3/4:1/4)	n=16	3.59 (1.04)	p>0.05 (0.714)
	(5/6:1/6)	n=16	3.73 (1.11)	
aW	(3/4:1/4)	n=16	0.9794 (0.006)	p>0.05 (0.689)
	(5/6:1/6)	n=16	0.9785 (0.006)	

In this study, the time of salting (8h or 6h) tested were not enough different to induce statistically significant differences between the two groups ($p>0.05$) (Table 3). The wet-salted samples (6h) treated with long drying and short smoking times (treatment II-d) had higher values for a_w and lower WPS values (Table 3). In both salting methods, the salt absorption by the fish increased, representing a critical point concerning limits to natural microbial a growth. The salt content had shown effects on other physicochemical characteristics of cold-smoked salmon, affecting the astaxanthin concentration of fillets and process yield (Birkeland and Bjerkeng, 2005). In this study, despite of the quantity of salt:sugar and length of salting applied to the fish according to their size in order to reach a safe percentage of 3-3.5% of salt in the water phase (Huss, 1994), this percentage was not always reached (Table 3) and some samples had a salt percentage lower than the desired content (3.5%). It is interesting to mention that, even in this laboratory experiment, with a controlled small numbers of fishes to process (almost no time differences in the application of salt over the whole set of samples), the salt percentage varied quite a lot, which indicated a potential problem in terms of safety

at industrial smoking level where numbers of samples are high and variation is likely to be even greater. In general, the a_w of all the samples, however do not reveal significant differences (Table 3).

4.3.3.2 Microbiological behaviour of cold-smoked salmon-trout with respect to different salt:sugar salting proportions

The numbers in Figure 1 reflect the evolution of different groups of microorganisms during each five weeks of storage in relation to salting type and time and salting/drying/smoking and packaging process.

Regardless of the salting/smoking/packaging process, the samples with a high proportion of sugar in the salt mixture (salt:sugar|3/4:1/4) showed an increase in microbial numbers during storage (Figure 1, blue points). The wet salting process seems to induce more variability in samples (higher standard deviation) and higher microbial counts. As mentioned above, there were considerable differences on microbial content regarding the salting method (dry and wet) used (Figure 1). However, in relation to specific salting/smoking/packaging conditions, the brined samples showed interesting results, such as a decrease in *Enterobacteriaceae* and H_2S-producing bacteria counts in brined (8h) MAP samples (Figure 1). In general, cold-smoked samples with low sugar salt mixture concentrations, dry salted (8h) and smoked by short drying and long smoking (Group I) had low levels of microorganisms, particularly in conjunction with VP or MAP (Figure 2).

Table 3 – Physicochemical characteristics of different groups of salted/smoked salmon-trout fillets

Smoking process	Sub-group treatment	Salting process	Salt:Sugar proportion	Cold-smoked fillets	Weight loss (%)	NaCl in water phase (%)	aW
Group I	Ia	Drysalting, 8h	(3/4:1/4) (5/6:1/6)	n=4	12.28d 11.75d Average 12.02 (1.10)c	4.95 3.50 Average 4.22 (1.36)c	0.976 (0.008)a
	Ib	Drysalting, 6h	(3/4:1/4) (5/6:1/6)	n=4	10.38bcd 11.27cd Average 10.83 (0.77)bc	3.53 4.15 Average 3.84 (0.62)bc	0.978 (0.004)abc
	Ic	Brining, 8h	(3/4:1/4) (5/6:1/6)	n=4	8.64abcd 6.66ab Average 7.65 (1.18)a	3.90 3.91 Average 3.90 (1.02)bc	0.978 (0.006)abc
	Id	Brining, 6h	(3/4:1/4) (5/6:1/6)	n=4	8.44abcd 5.76a Average 7.10 (1.94)a	2.58 4.15 Average 2.65 (0.76)ab	0.985 (0.004)bc
Group II	IIa	Drysalting, 8h	(3/4:1/4) (5/6:1/6)	n=4	10.0abcd 9.25abcd Average 9.63 (0.63)b	4.27 5.18 Average 4.72 (0.56)c	0.973 (0.004)a
	IIb	Drysalting, 6h	(3/4:1/4) (5/6:1/6)	n=4	10.0abcd 9.45abcd Average 9.73 (0.46)b	3.59 4.42 Average 4.00 (1.07)bc	0.977 (0.007)ab
	IIc	Brining, 8h	(3/4:1/4) (5/6:1/6)	n=4	6.5ab 6.90abc Average 6.700 (1.14)a	3.69 3.51 Average 3.60 (0.35)abc	0.979 (0.002)abc
	IId	Brining, 6h	(3/4:1/4) (5/6:1/6)	n=4	6.75ab 6.10ab Average 6.425 (1.56)a	2.23 2.47 Average 2.35 (0.58)a	0.987 (0.003)c

Group I – Smoked samples undergoing short drying and long smoking times. Group II – Smoked samples undergoing long drying and short smoking times. Columns with the different digit differ statistically ($p<0.05$).

Table 4 – Chemical and microbiological counts for different groups of samples during chilled storage of cold-smoked samples packed on VC and MAP

Group treatment	Week storage	Cold-smoked samples	APC	Average group	LAB	Average group	Enterobacteriaceae	Average group	H_2S-producing bacteria	Average group	pH	Average group	TMA	Mean group
Group I - VP	1	n=16	2.69 (1.13)	4.82 (1.90)a	1.31 (0.64)	1.92 (1.23)a	2.55 (1.19)	4.14 (1.65)b	1.13 (1.37)	1.83 (1.97)a	5.93 (0.15)	6.08 (0.19)a	3.36 (1.62)	9.11 (11.34)a
	2	n=16	4.16 (1.24)		1.37 (0.55)		3.75 (1.41)		1.23 (1.00)		5.93 (0.15)		3.19 (1.88)	
	3	n=16	4.83 (1.44)		1.43 (1.45)		4.49 (1.52)		1.48 (1.81)		6.22 (0.08)		7.07 (7.90)	
	4	n=16	5,92 (1.54)		2.15 (1.11)		4.68 (1.84)		0.81 (1.38)		6.04 (0.15)		17.58 (16.9)	
	5	n=16	6.50 (1.54)		3.33 (0.92)		5.23 (0.83)		4.49 (1.62)		6.25 (0.09)		14.36(12.86)	
Group I - MAP	1	n=16	3.84 (0.85)	5.46 (1.76)b	nd	1.96 (1.30)a	1.59 (0.85)	3.29 (1.97)a	nd	1.73 (2.13)a	5.97 (0.09)	6.04 (0.11)a	13.00 (12.66)	12.88 (14.28)a
	2	n=16	4.94 (1.24)		1.35 (1.33)		2.30 (1.68)		1.09 (1.46)		5.97 (0.09)		7.55 (12.11)	
	3	n=16	5.53 (1.37)		nd		3.20 (1.70)		nd		6.12 (0.08)		8.58 (7.08)	
	4	n=16	6.50 (1.83)		2.57 (0.99)		4.67 (1.69)		2.37 (2.53)		6.06 (0.11)		22.69 (21.76)	
	5	n=16	6.50 (1.83)		nd		4.67 (1.97)		nd		6.06 (0.11)		nd	
Group II - VP	1	n=16	2.69 (1.06)	5.41 (2.11)b	1.90 (0.85)	3.60 (1.80)b	2.03 (1.18)	4.69 (2.36)b	0.85 (0.58)	2.60 (2.60)a	6.15 (0.12)	6.27 (0.14)b	2.70 (1.82)	30.92 (37.80)b
	2	n=16	4.55 (1.48)		2.80 (1.41)		3.65 (2.08)		1.38 (1.10)		6.15 (0.12)		7.57 (5.95)	
	3	n=16	5.58 (1.34)		3.86 (1.63)		4.98 (1.70)		1.12 (1.18)		6.38 (0.07)		30.89 (28.67)	
	4	n=16	6.62 (1.48)		4.09 (1.62)		5.98 (1.82)		2.29 (2.07)		6.35 (0.08)		45.99 (43.47)	
	5	n=16	7.62 (0.81)		5.32 (1.35)		6.82 (1.46)		6.26 (2.70)		6.34 (0.06)		79.66 (37.20)	
Group II - MAP	1	n=16	3.96 (1.09)	6.07 (1.92)c	nd	1.96 (0.75)a	2.59 (0.48)	5.46 (2.07)c	nd	1.68 (2.75)a	5.97 (0.09)	6.04 (0.11)a	11.61 (14.26)	28.39 (37.38)b
	2	n=16	4.90 (1.01)		1.70 (0.66)		4.32 (0.30)		0.82 (1.40)		5.97 (0.09)		11.73 (9.52)	
	3	n=16	6.61 (1.20)		nd		5.78 (1.05)		nd		6.11 (0.08)		55.32 (60.55)	
	4	n=16	7.45 (1.60)		2.22 (0.77)		7.30 (1.34)		3.53 (3.48)		6.06 (0.11)		40.87 (36.02)	
	5	n=16	7.45 (1.60)		nd		7.30 (1.34)		nd		6.06 (0.11)		nd	

Group I – Smoked samples undergoing short drying and long smoking times; Group II – Smoked samples undergoing long drying and short smoking times

Columns with the different digit differ statistically ($p<0.05$).

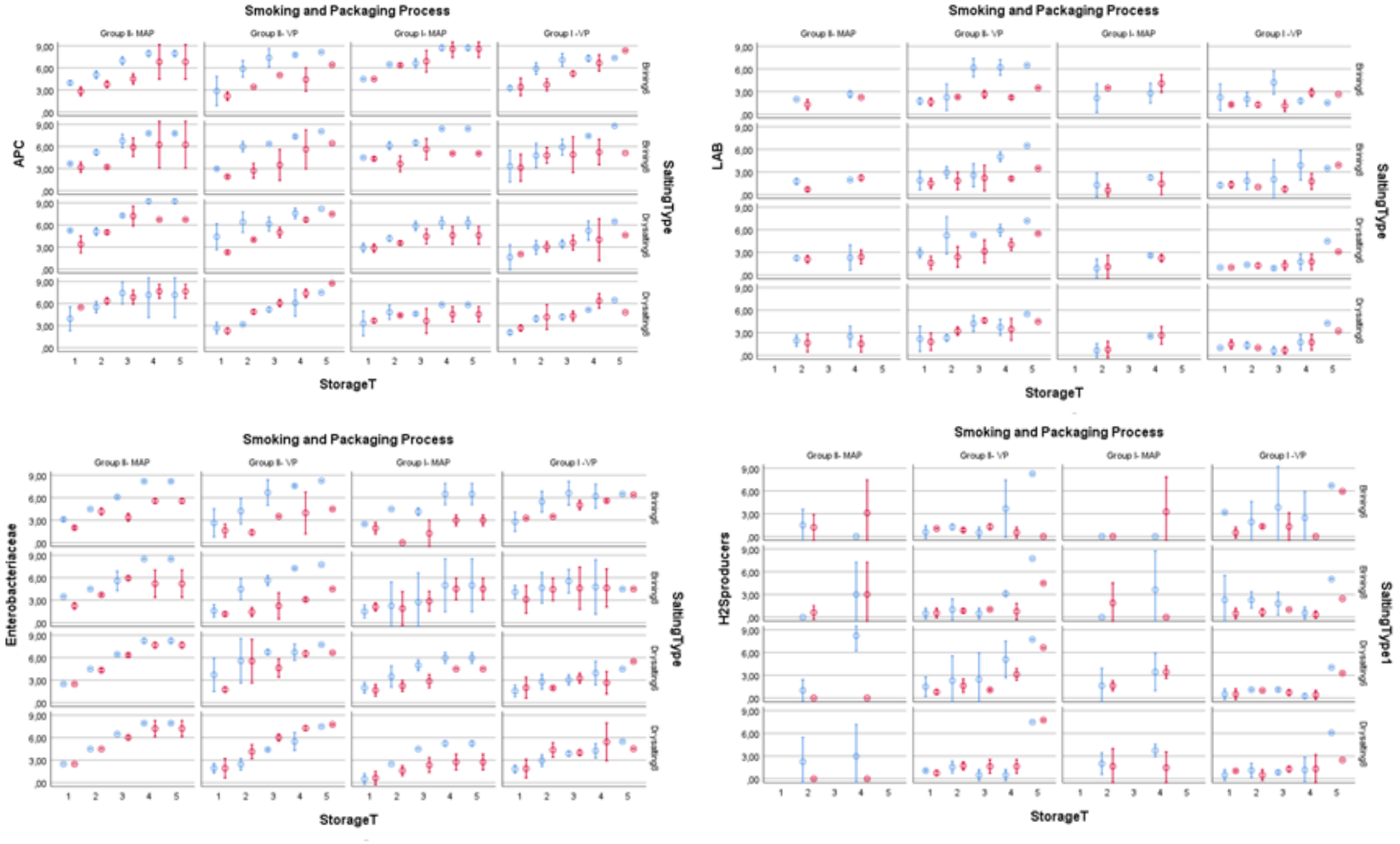

Figure 1. – Microbiological evolution (Log CFU/g) during chilled storage conditions of different cold-smoked salmon trout samples stored in VP and MAP. (Group I – Smoked samples undergoing short drying and long smoking times; Group II – Smoked samples undergoing long drying and short smoking times

4.3.3.3 Chemical and microbiological characteristics of cold-smoked salmon trout stored in VP or MAP

The chemical and microbiological characteristics of packed salmon trout Group I (short drying and long smoking) and Group II (long drying and short smoking) in combination with VP or MAP conditions, are shown in Table 4. Comparing these two groups of samples stored in VP, Group II – VP had higher microbiological numbers for APC, LAB, *Enterobacateriaceae* and H_2S-producing bacteria and, after 4 weeks of storage, an increase of 0.70 Log CFU/g, 1.94 Log CFU/g, 1.30 Log CFU/g and 1.48 Log CFU/g respectively. Additionally, pH and TMA values increase, respectively, to 6.35 and to 45.99 mg.100 g-1 of fish ($p<0.05$) within the same group (Table 4). The evidence of high numbers of LAB is in agreement with results already published by Tomé *et al*., (2009) that showed the possibility of selecting LAB by application of different smoking processes. However, the results of this study showed a significant increase in TMA values, pH, APC and LAB counts ($p<0.05$) and an increase in numbers of *Enterobacteriaceae* and H_2S-producing bacteria (Table 4), revealing the limited application of the long drying/short smoking process with respect to quality and safety, and emphasizing the importance of attaining a sufficient level of smoke in the process. Despite the effects of long drying's microbiological status, and the improvement in the product's flavour and organoleptic characteristics, the presence of a significant concentration of smoke in the product seems to be an important factor for inhibiting/controlling and selecting microorganisms. Interaction between phenols and salt has been studied by Thurette *et al*. (1998) who showed that an inhibitory effect on *L. monocytogenes* is dependent on salt concentration, i.e. a mixture of 0.002% phenol and 2 or 3% NaCl had no effect, but the same phenol concentration with 4% of NaCl

led to a significant reduction in growth. Also, Leblanc *et al*., (2000) reported similar results in *S. putrefaciens* in dry salted cold-smoked salmon, demonstrating osmotic shock on the cells caused by salt and bacteria's subsequent greater sensitivity to phenols. These parameters in industrial smokeries can differ significantly, requiring close and tailored control of salting/drying/smoking processes to guarantee product safety and stability.

Under the tested conditions, and as shown in Table 5, the bacterial strains isolated from the group of samples that underwent long drying and short smoking times (Group II –VP) showed 48.9% positive results for TMA production in comparison with 29.8% positive results in Group I – VP. A higher percentage of this group of bacteria were linked to Group II – MAP (70.1%) (Table 5). These bacteria probably belong to a mixture of Gram negative bacteria linked to specific spoilage microorganisms, such as *Sh. putrefaciens* and *Ph. phosphoreum* (the former, with lower proportions in VP and the second representative in MAP conditions), and a significant number of strains of *Enterobacteriaceae* and others, as shown in Table 4 and in Table 6, represented by the *Serratia/Hafnia* genera.

Higher positive results for bacteria were also seen for proteolytic and lipolytic activity linked to Group II – VP (55.3% and 27.7% respectively) (Table 5). On the other hand, in challenge experiments with cold-smoked salmon, Joffraud *et al*., (2006) showed that *Lactobacillus sakei*, *L. alimentarius* and *L. farciminis* grew faster than *S. putrefaciens*, *P. phosporeum*, *B. thermosphacta* and *Serratia liquefaciens*, indicating LAB's responsibility in the spoilage process. LAB can be present in higher percentages (60%) at the end of shelf life in some products, particularly *Carnobacterium* spp. and *Lactobacillus* spp. Leroi (2010) also showed that amino acids are catabolized by carnobacteria into aldehydes and alcohols, whilst cysteine and arginine can be

metabolized into H_2S and NH_3 respectively, responsible for sulphurous and ammonia off-odours. Citrate is a precursor of diacetyl, responsible for dairy and butter-like odours and often produced by *Leuconostoc* and *Lactococcus* species.

Microbiological and chemical patterns for samples stored in VP (Group I – VP) and in MAP (Group I – MAP), both submitted to the short drying and long smoking process, are shown in Table 4. A significant increase in APC and a decrease in *Enterobacteriaceae* numbers were observed ($p<0.05$), and a small increase in LAB and H_2S-producing bacteria were observed in MAP samples after 3 weeks of storage. The growth-inhibitory effects of MAP vary for different microorganisms, not only reducing microbial growth, oxidative reactions, and metabolic activity of the foods, but also imposing changes in the composition of microbial flora, improving the growth of some microorganisms.

MAP can discourage the growth of aerobes (such as *Pseudomonas*, *Psychrobacter*, and *Shewanella*) and encourage growth of facultative anaerobes (such as *Enterobacteriaceae*, *Aeromonas*, *Photobacterium*, *Brochothrix*, and lactobacilli) and anaerobes (Erkmen and Bozoglu, 2008a). Carbon dioxide's mode of action on microorganisms depends not only on the concentration, but also on the a_w, acidity (pH), temperature parameters, and numbers and age of microorganisms (Day, 2008; Erkmen and Bozoglu, 2008a). Due to rapid cellular penetration, the solubility of carbon dioxide in water to form H_2CO_3, reducing pH (environmental and cytoplasmic acidification). In this study, both groups of samples submitted to MAP (Group I and Group II) had similar acidity, regardless of the drying/salting/smoking processes used. The pHs observed vary from 5.97, initially, to 6.06 after 5 weeks' storage (Table 4). The results suggested that MAP promoted an acid environment, inducing the growth of acid-tolerant bacteria and, in anaerobic conditions, the presence of LAB and other Gram-

negative bacteria with the potential to cause spoilage. In this study, the LAB strains were analysed by electrophoresis protein patterns (SDS_PAGE), revealing similarity with *Lactococcus lactis lactis* especially in Group II – MAP of samples (results not shown). In fact, the results revealed an increase in TMA levels in Group II – MAP samples, statistically different from the Group I – MAP samples ($p<0.05$) (Table 4), but not from the Group I – VP samples ($p>0.05$). These results reflect the efficacy of prior combined treatment of drying/salting/smoking on microbiological selection, rather than type of packaging at the end of process. This effect was also demonstrated in VP samples. The results presented in Table 5 demonstrate the high percentage of bacteria that reduce TMAO to TMA in Group II samples (70.1% and 48.9%), by comparison with the 24.7% and 29.8%, observed in Group I samples. These findings demonstrated that a short drying and long smoking process (Group I) is preferable to a long drying and short smoking process (Group II) this last also showing higher percentage of bacteria positive for proteolytic and lipolytic activity, which were more relevant in VP samples (Table 5).

Table 5. Positive reaction of different bacterial isolated from different groups of cold salmon trout in different agar media for H_2S-producing bacteria, TMA reaction, proteolytic (AP) and lypolitic activity

Packaging	Salting process	H_2S		TMA		AP		AL		Total	
		Group I	Group II	Group I	Group II	Group I	Group II	Group I	Group II	Group I	Group II
VP	Dry Salting, 8h	0/12	1/13	1/12	5/13	5/12	5/13	0/12	2/13	6/12 (50.0%)	13/13 (100.0%)
	Dry Salting, 6h	1/12	0/15	3/12	7/15	6/12	10/15	1/12	4/15	11/12 (91.7%)	21/15 (140.0%)
	Brining, 8h	2/10	0/10	4/10	5/10	5/10	5/10	3/10	3/10	14/10 (140.0%)	13/10 (130.0%)
	Brining, 6h	5/13	0/9	6/13	6/9	5/13	6/9	2/13	4/9	18/13 (138.5%)	16/9 (177,8%)
	Total	8/47 (17,0%)	1/47 (2,1%)	14/47 (29,8%)	23/47 (48,9%)	21/47 (44,7%)	26/47 (55,3%)	6/47 (12,8%)	13/47 (27,7%)	49/47 (104,3%)	63/47 (134,0%)
MAP	Dry salting, 8h	4/20	2/21	3/20	16/21	10/20	13/21	2/20	0/21	19/20 (95.0%)	31/21 (147.6%)
	Dry salting, 6h	11/23	3/28	8/23	21/28	8/23	14/28	3/23	3/28	30/23 (130.4%)	41/28 (146.4%)
	Brining, 8h	10/20	0/15	6/20	7/15	8/20	7/15	1/20	1/15	25/20 (125.0%)	15/15 (100.0%)
	Brining, 6h	12/22	2/23	4/22	17/23	8/22	12/23	0/22	2/23	24/22 (109.1%)	33/23 (143.5%)
	Total	37/85 (43,5%)	7/87 (8,0%)	21/85 (24,7%)	61/87 (70,1%)	34/85 (40,0%)	46/87 (52,9%)	6/85 (7,0%)	6/87 (6,9%)	98/85 (115,3%)	120/87 (137,9%)

Group I – Smoked samples submitted to short dry and long smoke

Group II – Smoked samples submitted to long dry and short smoke

Table 6. Biochemical characterization of bacteria isolates from group of *Enterobacteriaceae*

		EPM culture médium				Kligler culture medium				MILI culture medium			
		Glucose	H_2S	Urease	Desaminase	Lactose	Glucose	H2S	Gas	Mobility	Indol	Lysine - Descarboxylase	Number of colonies (%)
Group I - VP	*Serratia*/*Hafnia*	+	-	-	-	-	+	-	+/-	+	-	+/-	15/27 (56%)
	Enterobacter	+	-	-	-	+	+	-	+	+	-	+/-	6/27 (22%)
	Shigella	-	-	-	-	-	+	-	-	-	-	-	1/27 (4%)
	Others	Different combinations											5/27 (19%)
Group II- VP	*Serratia*/*Hafnia*	+	-	-	-	-	+	-	+/-	+	-	+/-	10/14 (71%)
	Klebsiella	-	-	+	-	-	+	-	-	-	+/-	-	1/14 (7%)
	Others	Different combinations											3/14 (21%)
Group I - MAP	*Serratia*/*Hafnia*	+	-	-	-	-	+	-	+/-	+	-	+/-	24/36 (67%)
	Enterobacter	+/-	-	-	-	+	+	-	+/-	+	-	+/-	1/36 (3%)
	E. coli	-	-	-	-	-	+	-	+/-	+	+	+	1/36 (3%)
	Providencia	-	-	-	-	-	+	-	-	-	+	+	1/36 (3%)
	Others	Different combinations											9/36 (25%)
Group II - MAP	*Serratia*/*Hafnia*	+	-	-	-	-	+	-	+/-	+	-	+/-	23/39 (59%)
	Enterobacter	+/-	-	-	-	+	+	-	+	+	-	+/-	7/39 (18%)
	E. coli	-	-	-	-	-	+	-	+	+	+	+	1/39 (3%)
	Shigella	-	-	-	-	-	+	-	-	-	-	-	1/39 (3%)
	Others	Different combinations											7/39 (18%)

4.3.4 Conclusions

Several studies indicated that salting and smoking procedures have an important role of minimizing the risk of foodborne hazards and the spoilage of cold-smoked salmon (Lovdal, 2015; Martinez *et al*., 2012). Tocmo *et al*. (2014) reported that the final product's stability was dependent, among other factors, on the type of salting, smoking and drying conditions in conjunction with packaging and storage temperatures.

In this study, the influence of different curing-smoking processes was investigated, especially type and time of salting (dry and wet salting for different salting periods) as well different salt: sugar proportions. The results reveal, in general, that dry salting encouraged a high average weight loss in fillets compared to brining, with a resulting impact on microbiological status. Overall, dry salting is preferable to the brining process for reducing numbers of microorganisms in cold-smoked salmon trout, in combination with short drying, long smoking and packed in VP. The presence of LAB, *Enterobacteriaceae*, H_2S-producing and bacterial producers of TMA was influenced by the technological processes applied during the curing process in relation to type of salting and the length of the smoking process. Process Group II (long drying and short smoking) provides the best solution for promoting LAB growth, especially in the combination of dry salting with a salt:sugar proportion of 1/4 of sugar and packaged in VP or MAP. In this case, interest in the microbiological characteristics of other groups also suggested an increase in *Enterobacteriaceae*, H_2S-producing bacteria and TMA bacteria producers. In general, cold-smoked samples with a low sugar concentration, dry salting (8h) and short drying and long smoking times (Group I) had low levels of microorganisms, particularly in conjunction with VP or MAP. Results also showed that the type of packaging, VP or MAP, also influenced the microbiological

ecology of cold-smoked salmon trout, with the greatest impact on the samples in Group II (long drying and short smoking times).

CHAPTER 5

Conclusions and final remarks

Worldwide consumers demand high-quality and safe food products. Concerns about safety of ready-to-eat vacuum-packed cold-smoked fish products increased because these products have been associated with listeriosis outbreaks and an image of 'unsafe' products was created. The dynamic of health and surveillance entities, as well as the scientific community have contributed to the production of scientific and technical documents with public interest. Typifying the main phases related with cold-smoked fish production and considering the work areas which influence the quality of cold-smoked fish products, three areas are identified: 1) Origin and quality of the raw material; 2) Technological procedures applied during the curing preservation process and 3) Package and storage conditions.

One of the conclusions of this study concerns the great variability of cold-smoked fish samples, due to previous phases of processing, inducing different microbial and chemical characteristics of end-products. This difference is evidenced at initial time week of chill storage, maintaining during storage or less pronounced later storage of cold smoked fish. The storage conditions are still associated with fluctuations in refrigerated system (time and temperature) and practices from smoke producers and from commercial market. This conclusion is supported in this study by differences on microbial quality of samples obtained at Portuguese points of sale, evidencing differences in shelf-life, as well in the microbiological composition of the cold-smoked samples. In addition, some of these cold-smoked samples are at the limit of the allowed microbial load for RTE products, even before reaching shelf life´s limit. The dominance of LAB and *Enterobacteriaceae* was observed in cold-smoked salmon and salmon-trout products.

Some microorganisms are able to produce biogenic amines by decarboxylation of amino acids, and some spoilage bacteria of cold smoked products can be active in the

production of biogenic amines. The results demonstrated that some bacterial strains isolated from cold-smoked fish belonging to the group of LAB and *Enterobacteriaceae* were positive for tyramine production and less for histamine production. Strict hygiene during processing and handling is a prerequisite for the cold-smoked fish quality and shelf-life determination. The abuse-temperatures of storage during cold-smoked processing are considered relevant having impact in the quality of end-product. The microbiological growth and selection are temperature dependents, as well as the salting/smoking process. The temperature of storage is considered a critical control point for storage of cold-smoked fish that should be stored and distributed at temperatures less than 4.4°C (or frozen) (HACCP recommendation FDA, 2001).

Considering the presence of the pathogen *L. monocytogenes* and the reduction of microbiological numbers in raw fish, the application of gaseous ozone on fresh fillets salmon-trout surfaces resulted in a small reduction of *L. innocua* 2030c (less than 1 Log_{10} CFU/g) and a very low number of total viable counts after 3 weeks of storage (less than 1 Log_{10} CFU/g). The efficacy of gaseous ozone exposure on microbiological reduction during cold-smoking process was low, but it can be a very promising agent as a disinfectant, used as ozonized water or as an aerosol (spray).

The step of freezing cold-smoked vacuum-packaged salmon–trout was studied in this work. This practice is recommended to kill parasites which may be present in fresh fish, particularly in the wild fish (HACCP recommendation, FDA, 2001). The freezing can be applied in a previous step to raw fillets or to smoked fillets or at the end product in a vacuum package. In this study, the previous freezing step showed effects on microbial load in cold-smoked samples, when compared to non-frozen cold-smoked samples. This evidence was not surprising, since the thermal stress, together with the previous salting/smoking treatments applied, allowed changes in muscle proteins,

providing potential effects on microbial growth. The proteins denaturation is temperature dependent, the ice crystals are deposited in cells and are affected by time-temperature process. During the salting process, the uptake of salt and the removal of water from fish can induce changes in myofibrillar proteins, being able to cause changes in texture and yield of the process. In this study, the DSC thermograms of myosin and actin were changed after salting/smoking process, evidencing changes of muscle structure during processing.

For a WPS target, dry salting or wet salting did not show the same yield performance and, in some cases, the WPS required percentage. The salt removal in the product depends on the method applied, so it will not be possible to obtain a final product with similar physiochemical characteristics, utilizing the same time of salting. These two salting processes should be treated differently in the process (e.g. salting conditions, control parameters, e.g. time, temperature) considering the physicochemical desirable effects on end-product and microbiological quality and safety. Different proportions of sugar in the salting process (salt:sugar|3:1) induced microbiological growth in cold-smoked samples, and can be suitable for the increase of the levels of desirable microorganisms as LAB. The salting process is considered a critical control point concerning cold-smoking processing, indicated of 3,5% of WPS for end product, that must be attained with type of packaging and storage temperature control (HACCP recommendation, FDA, 2001). This step is especially important to inhibit the growth and toxin formation of *C. botulinum* in the final product, as well as in combination with other parameters, such as acidity (pH), salt and water activity.

The main purpose of cold smoking <30ºC is developing desirable organoleptic characteristics in the end-product. The action of smoking on the microorganisms is fundamentally due to the multiple compounds present in the smoke, and not only by the temperature. The low temperatures of smoking permit the survival of a significant microbiota on the fillets and can represent a competitive natural microorganisms for restrict *C. botulinum* growth. The inadequate temperatures and time control could exacerbate the hazards (for *C. botulinum* growth and toxin production and for *L. monocytogenes*), being considered a critical control of point. The short-term cold smoking (<24h) is recommended by the guidelines of AFDO (1991).

The research presented here demonstrated the smoking process characterized by long drying and short smoking times (Group II – Dry 6h and Smoke 2h), which encouraged a general increase on microbiological numbers especially in LAB levels. However, a negative effect on the samples regarding microbiological quality was observed, by significant increase of spoilage microorganisms (*Enterobacteriaceae* and H_2S-producing bacteria) and significant levels of TMA in end-products. *Serratia/Hafnia* and *Enterobacter* genera were representative of the *Enterobacteriacea* group in all cold-smoked salmon-trout, which can also contribute for production of biogenic amines. Despite the positive effects of long time of smoke in samples (Group I – Dry 2h and Smoke 6h), dry salted samples (8h) presented the best combination for microbial reduction, either in VP or MAP. MAP represents an alternative of packaging to VP, reducing the microbiological activity of some spoilage bacteria. The CO_2 is the most important gas used in MAP by their bacteriostatic properties. The effectiveness of the gas is always conditioned by the storage temperature, resulting in an increase when the temperature decreases. The inhibition of microorganisms is determined by the concentration of dissolved CO_2 in the product, resulting on drop of pH in product

surfaces. The packaging is not considered a critical control of point, since is not possible to label safety into a product. The identification of temperature and time of storage for safety is a critical control of point regarding the biogenic amine production in ‘scombrotoxin-susceptible species’ as well as it is important for *C. botulinum* growth and toxin formation and also to reduce the growth rate of *L. monocytogenes*.

CHAPTER 6

Proposals for future work

Considering the results presented in this study, the cold smoking process should be reviewed mainly on the productive process and studies on the details of microbiological contamination and developments during storage in general.

Thus, the following research will be proposed for future work:

1) Studies on the efficacy of application of different sanitizing agents on environmental smoke plants areas (e.g. ozone/ozonised water);
2) Studies on reduction of microorganisms from raw fish: application a step washing in whole fresh fish or fillets, with ozonized water (or in spray);
3) Experiments with cold smoking processing, involving new "hurdle concept" preservative effects:
    3.1 Studies on salting process e.g. combined treatments with brine (as previous a prior treatment) followed by a light dry salting treatment, in attempts to minimize WPS variation within and between fillets;
    3.2 Studies on the effects of vacuum and modified atmospheres packaging in combination with different temperatures of storage ('superchilled' temperatures' and temperatures less than 4.4) on the microbiota of different cold-smoked fish products as obtained in results of 3.1.
    3.3 Detailed studies on the elective effects of the various salting/drying/smoking regimes on the selection of inhibitory/anti-listerial LAB.

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